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STUDIES ON THE MECHANISM FOR SUGAR ABSORPTION FROM RAT INTESTINE, WITH ESPECIAL REFERENCE TO THE SORT OF THE PHOSPHATE ESTERS FORMED DURING THE ABSORPTION

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(Received for publication, January 24, 1957)

Many investigators (*1–7*) noted the fact that the amount of phosphate esters accumulated in the intestinal mucosa during absorption of hexoses and the rate at which hexoses were absorbed in the mucosa were both reduced after administration of phlorizin, monoiodoacetate, or dinitrophenol. They have advanced the theory that the active transport of hexoses through the intestinal mucosa is made possible by their intermediary phosphorylation and that the process of their phosphorylation takes place in the hexose-absorbing mucosa.

Admitting that it is true, the particular kind of hexose phosphate that will be formed from the hexose absorbed is very difficult to be determined, because the conventional method of fractionation in which barium salt is used is inadequate for an exact quantitative or qualitative determination of each individual sugar phosphate in the mucosa.

The object of the present research was to determine the particular sort of the major esters of hexose absorbed, using glucose, galactose and fructose along with P^{32} by means of paper chromatography and radioautography. Elucidation of the enzymic splitting and the intestinal absorption of the esters was another object of the research.

METHODS AND PROCEDURES

Fractionation of Acid Soluble Phosphate—TCA[1] extract of intestinal mucosa prepared by the usual method was separated into three fractions of acid soluble phosphorus compounds by the use of the solubilities of the barium salts according to LePage's (*8*) method.

1) Trichloroacetic acid.

Determination of Phosphate—Barium in each fraction was removed by application of ice-cold 0.1 N H_2SO_4 and the Ba-free fractions were respectively deorganised with 2 ml. of perchloric acid. In the case of the ester separated by the paper chromatography, the sections of the paper corresponding to the spots identified on the radioautogram were ashed with 2 ml. of perchloric acid. The phosphorus content in the solution thus obtained was estimated by the colorimetric method of Barton (*9*).

Determination of Radioactivity—P^{32} in the solution of ash was precipitated in the form of $Mg(NH_4)P^{32}O_4$ by the addition of a carrier, and assayed with an end-window Geiger-Müller tube (Kobe Kogyo).

Paper Chromatography and Radioautography—The filter paper used (Toyo filter paper No. 131) had been washed previously first with 0.2 per cent ethylenediaminetetraacetic acid by the method of Eggleston and Hems (*10*) and then with 1 N HCl. The paper chromatography of the fractions of LePage was carried out according to Sekiguchi (*11*). Development was conducted at 0°±2°. The spots of authentic samples were located by spraying with the molybdate reagent of Hanes (*12*), followed by heating at 85° according to Axelrod (*13*), and their R_f values were compared with those obtained by radioautography.

The radioautography was continued in a cacette for a period ranging from one to two weeks in depending on the intensity of the radioactivity of the spot, as in the experiment of Grobbelaar and Steward (*14*).

Preparation of Extract of Intestinal Mucosa—The intestinal mucosa was homogenized by the use of 3 times its volume of veronal acetate buffer (pH 6.8), and centrifuged for 10 minutes at low speed. The supernatant fluid was removed carefully by suction in a refrigerator.

Determination of Glycogen Content in the Mucosa—Intestinal mucosa was scraped away and submitted to glycogen determination by the method of Somogyi (*15*).

Preparation of Purified Intestinal Phosphatase—The enzyme was extracted from rat intestinal mucosa and purified by the method of Morton (*16*).

Samples Used for Determination of R_f Values or Other Experiments—G-1-P[2] (K-salt) synthesized by the method of Hassid (*17*), and commercial samples from L. Light & Co., Ltd. and Nutritional Biochemicals Corporation. G-6-P[3] (Ba-salt) prepared according to Horecker (*18*) and commercial sample from Nutritional Biochemicals Corporation. F-1,6-diP[4] (Ca-salt) and β-glycerophosphate (Na-salt) were commercial samples from E. Merck and Katayama Chemicals respectively.

Male and female albino rats weighing 150 to 200 g. were used throughout the present experiments with an exception of rabbits used in an experiment.

Experiments in vivo—The abdominal cavity of rat kept fasting for the last 24 to 48 hours was opened under ether anesthesia. For making of radioautogram, 2 ml. of a 20 per cent solution of hexose containing $NaH_2P^{32}O_4$ (316×10^3 c.p.m.)—in the case of

2) Glucose-1-phosphate.

3) Glucose-6-phosphate. These synthesized esters were kindly given by Dr. T. Kawachi and Dr. A. Tanaka (in our laboratory) respectively.

4) Fructose-1,6-diphosphate.

the determination of glycogen contents, the hexose solution without radio isotope, was injected into the gastric cavity. The saline solution instead of the hexose solution was given in control animals. The abdomen was next closed and the animal was kept in a cage for the following one hour. The intestine was removed, cut open, the intestinal contents washed out thoroughly with ice-cold saline, and the intestinal mucosa was scraped off from the muscular layers on a glass plate.

For the estimation of absorption rate of hexose or hexose phosphate from the intestine, the upper half of the jejunum was ligated up at two places about 15 cm. apart. Two ml. of hexose or hexose phosphate in solution was injected into the loop. Just one hour afterwards, the loop was removed from the animal, the end part of the loop was cut open over a funnel, the contents of the loop were flushed out with the saline solution.

Experiments in vitro—For the experiment on interconversion of glucose phosphate, 2 ml. of the extract of intestinal mucosa was incubated at 37° with 0.06 *M* NaF, 0.03 *M* $MgCl_2$ and 0.01 *M* G-1-P or G-6-P. The pH of the mixture was adjusted to 7.2 with veronal acetate buffer, the final volume being 5 ml. After 5 minutes' incubation, the reaction mixture was cooled rapidly in an ice water, and the same volume of ice-cold 10 per cent TCA was added. The phosphorus compounds in the TCA extract were fractionated according to LePage and estimated by the method of Barton. G-1-P in the Ba-soluble alcohol-insoluble fraction was determined by hydrolyzing with 1 *N* HCl for 7 minutes at 100°. Moreover the exclussive presence of G-1-P and G-6-P in the fraction was confirmed by paper chromatography.

In the experiment on the intestinal phosphatase, the enzyme purified according to Morton was incubated with a substrate at 37° for 5 minutes, the pH of the medium being adjusted to 9.5 with veronal-HCl buffer. The reaction was stopped by the addition of an equal volume of 10 per cent TCA.

RESULTS

The amount and radioactivity of the esters determined by the fractional and colorimetrical methods, formed in the intestinal mucosa during absorption of hexose were shown in Table I. It was observed that both of acid labile phosphate formed during absorption of fructose increased although the results were essentially identical with those obtained by Ota and Shibata (*7*) in our laboratory.

As has been stated, this procedure provided no satisfactory information available for determination of the individual esters formed in the mucosa while hexose was being absorbed there. Paper chromatography and radioautography were therefore carried out.

The R_f values of the esters were somewhat lower universally in comparison with those obtained by Sekiguchi (*11*). The esters accumulated in the intestinal mucosa during the absorption of hexoses,

Table I

Changes of the Phosphates in the Intestinal Mucosa of Rat during Absorption of Hexose

Two ml. of 20 per cent solution of hexose containing $NaH_2P^{32}O_4$ (316×10^3 c.p.m.) was administered. One hour afterward, TCA extract of intestinal mucosa was prepared and fractionated according to LePage. Phosphorus content in each fraction was determined by the colorimetric method of Barton. Radioactivity was estimated by Geiger-Müller tube.

1) *mg.* P per 100 *g.* of dry tissue weight

Substance applicated	TCA extract		Ba-insol. fraction			Ba-sol. alcohol-insol. fraction		Ba-sol. alcohol-sol. fraction
	Inorg. P	Total P	Inorg. P	$\Delta 7'$P	Org. P	$\Delta 7'$P	Org. P	Total P
Glucose	138.8	352.7	133.7	27.9	40.8	21.6	134.1	9.7
Galactose	162.5	360.2	151.9	22.7	40.8	32.4	150.7	6.2
Fructose	152.7	368.2	159.8	45.9	61.6	66.7	161.2	7.5
0.9% NaCl	147.4	269.4	130.7	16.5	30.6	11.9	78.5	8.9
2) Specific activity (c.p.m./*mg.* P)								
Glucose	16744	10629	16702	2402	1719	4549	5429	1942
Galactose	24118	15609	23735	2462	4250	4892	7382	3014
Fructose	21305	14670	21049	9078	10596	11967	12897	2679
0.9% NaCl	4402	3446	4401	2360	1764	2448	2870	1506

$\Delta 7'$ P shows acid labile phosphate liberated in *N* HCl, for 7 minutes, at 100°.

The above figures were obtained on an average of experiments with 3 (galactose and fructose) or 6 (glucose and 0.9 per cent NaCl) rats.

glucose, galactose and fructose were identified as follows (see Fig. 1, a, b):

Hexose absorbed	Esters formed
Glucose	G-1-P, G-6-P and F-1,6-diP.
Galactose	G-1-P, G-6-P, Ga-1-P?[5] and Ga-6-P?[6]
Fructose	G-6-P, F-1,6-diP, F-1-P?[7] and an unknown ester.

5) Galactose-1-phosphate.

6) Galactose-6-phosphate could not be decided owing to the lack of authentic samples.

7) Fructose-1-phosphate was contained in a Ba-soluble alcohol-insoluble fraction and sat near Ga-1-P on radioautogram. It should not be fructose-6-phosphate because, according to Sekiguchi, fructose-6-phosphate is highest in R_f among these hexose phosphate esters. It was probably F-1-P, though nothing conclusive can be said as no authentic sample was available.

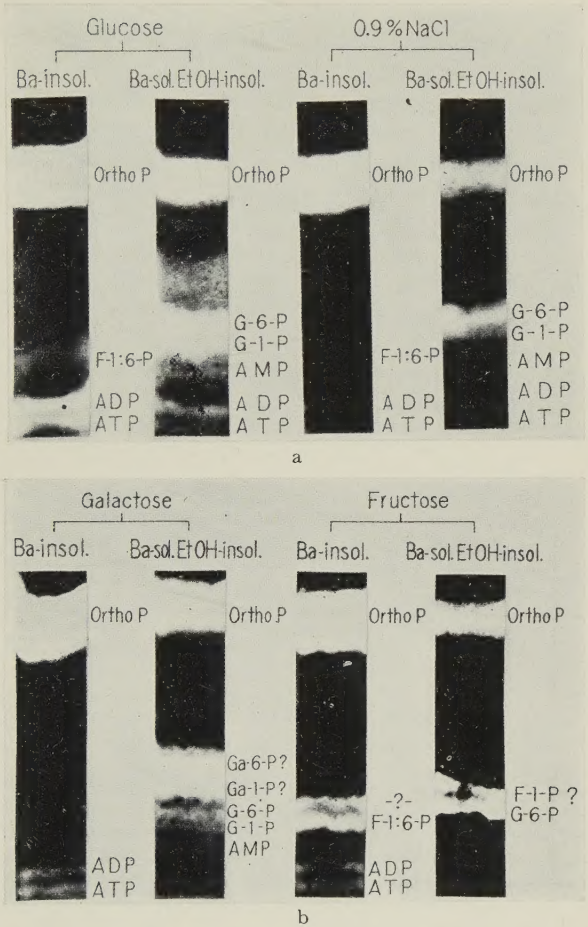

FIG. 1. The radioautograms of the esters accumulated in the intestinal mucosa of rats during absorption of hexose containing P^{32}.

The radioautography has been carried out using the paper chromatogram performed according to Sekiguchi, after fractionating TCA-extract of the mucosa by the method of LePage.

An ester located on radioautogram has been identified by comparing with the R_f of the authentic sample. The location of the spot of an authentic sample on the chromatogram was accomplished by spraying the molybdate reagent of Hanes, followed by heating at 85° according to Axelrod.

The amount and radioactivity of the phosphates separated by paper chromatography were determined as given in Table II.

It is presumable from the data in Table II that phosphoglucomutase may exist in the intestinal mucosa and that the enzyme may be establishing an equilibrium between G-1-P and G-6-P during the absorption of glucose. An experiment, therefore, was undertaken to elucidate whether such an equilibrium exists in the mucosa.

TABLE II

Analysis of the Hexose Phosphate Esters in the Hexose Absorbing Mucosa of Rat Separated by Paper Chromatography

Paper chromatography of the fractions obtained in the experiment described in Table I was carried out by the method of Sekiguchi. Phosphorus compound was determined according to Barton after ashing the section corresponding to the spot with 2 ml. of perchloric acid. Radioactivity was estimated by Geiger-Müller tube.

1) *mg.* P per 100 *g.* of dry tissue weight

Substance applicated	Ba-insol. fraction		Ba-sol. alcohol-insol. fraction*		
	Inorg. P	F-1, 6-diP	Inorg. P	H-1-P	H-6-P
Glucose	150.0	18.3	38.7	16.3	100.3
Galactose	171.4	13.6	41.2	18.3	127.3
Fructose	149.9	42.7	46.8	43.8	93.1
0.9% NaCl	158.0	9.7	24.7	8.1	54.0
2) Specific activity (c.p.m./*mg.* P)					
Glucose	19764	3897	7229	4545	4731
Galactose	28449	3613	8570	5919	5925
Fructose	24820	8953	8092	14214	15429
0.9% NaCl	4899	2351	2815	2675	1897

* H-1-P and H-6-P represent the esters corresponding to hexose absorbed. All data are means of 3 determinations.

The figures shown in Table III represent the results of interconversion of glucose phosphate after incubation with the extract of the mucosa. It will be seen evidently that G-1-P added to the reaction mixture was converted to G-6-P (G-1-P:G-6-P=1:17.6), on the contrary G-6-P added was hardly converted to G-1-P.

Cori and his collaborator (*19*) have reported that an equilibrium is attained in vitro between G-1-P and G-6-P at a molar ratio 1 : 19 at pH 7.0. The fact suggests that phosphoglucomutase does exist in the

mucosa, keeping the glucose phosphates in a state of equilibrium.

The effect of ATP on the phosphoglucomutase reaction is given in Table IV.

A very interesting phenomenon observed was that the addition of ATP accelerated the conversion of the added G-6-P to G-1-P, and that

TABLE III

Conversion of G-1-P to G-6-P, and Vice versa in the Extract of Intestinal Mucosa of Rat

The intestinal mucosa was homogenized with 3 times its volume of veronal acetate buffer (pH 6.8), centrifuged and then the supernatant fluid was used as the enzyme solution. Incubation period 5 minutes at 37° ; pH 7.0 (veronal acetate buffer) ; NaF, 0.06 *M* ; $MgCl_2$, 0.03 *M*. Phosphate compound was fractionated and estimated by the colorimetric method. Moreover their exclussive presence in a fraction was confirmed by paper chromatography, sprayed with the molybdate reagent.

1) Conversion of G-1-P to G-6-P

G-1-P added (P)	G-1-P remained (P)	G-6-P formed (P)
1080 μg	62 μg	1040 μg
"	56	1050
"	64	1035
"	54	1020
Average	59	1036

2) Conversion of G-6-P to G-1-P

G-6-P added (P)	G-1-P formed (P)	G-6-P remained (P)
1090 μg	58 μg	996 μg
"	61	1094
"	62	988
"	67	1077
Average	62	1039

this action of ATP became more marked with an increase in the amount of ATP added. On the other hand the conversion of added G-1-P to G-6-P was inhibited considerably on addition of ATP.

The amount of glycogen formed in the mucosa went on increasing while hexose was being absorbed, as shown in Table V, and essentially,

Table IV

Effect of ATP on the Conversion of G-6-P to G-1-P, and Vice versa in the Extract of Intestinal Mucosa of Rat and Rabbit in Vitro

The extract of the intestinal mucosa was obtained as described in Table III. Incubation period 10 minutes at 37° ; pH 7.0 ; NaF 0.06 *M*; $MgCl_2$ 0.03 *M*. G-1-P and G-6-P were determined as given in Table III.

1) Effect of ATP on the conversion of G-6-P to G-1-P (Rat)

ATP* added	G-6-P added (P)	G-1-P formed (P)	G-6-P remained (P)
mg.	*μg*	*μg*	*μg*
70	1085	650	410
50	"	518	622
30	"	406	668
0	"	67	1090

2) Effect of ATP on the conversion of G-1-P to G-6-P (Rabbit)

ATP* added	G-1-P added (P)	G-1-P remained (P)	G-6-P formed (P)
mg.	*μg*	*μg*	*μg*
70	1080	804	231
50	"	486	467
30	"	396	662
10	"	336	704
5	"	210	770
0	"	64	942

* Ba-salt prepared by the method of LePage (*20*), and applicated after converting to di-sodium salt.

Table V

Glycogen Content in the Intestinal Mucosa during the Absorption of Hexose

Two ml. of 20 per cent solution of hexose was injected into gastric cavity. Duration of the absorption was 1 hour. Glycogen content in the intestinal mucosa was determined by the method of Somogyi.

Hexose injected	Glycogen content	Glycogen increased
	*mg. per cent**	*mg. per cent**
Glucose	46.4	14.4
Galactose	50.8	18.8
Fructose	43.8	11.8
Control	32.0	—

* Based on the wet tissue weight.

Each value is the average of 8 determinations from 4 rats.

Table VI

Hydrolysis of Hexose Phosphate Esters by Purified Intestinal Phosphatase from Rats in Vitro

The enzyme was extracted from the intestinal mucosa and purified according to Morton and incubated with a substrate at 37° for 5 minutes pH 9.5 (veronal-HCl buffer). Phosphates in the TCA extract were fractionated and determined by the colorimetric method.

Substrate	Total P of substrate	Inorg. P liberated	
	μg	μg	%
G-1-P	880	550	62.5
G-6-P	880	232	26.4
F-1,6-diP	968	327	33.8
β-Glycero-P	905	386	42.7

All data are means of 4 determinations.

Table VII

Effect of 2,4-Dinitrophenol upon Absorption of Hexose or Hexose Phosphate from Rat Intestine

Two ml. of hexose or hexose phosphate solution was injected into the ligated intestinal loop. After the lapse of 1 hour, the contents of the loop were flushed out, and the phosphorus compounds in the flushings were fractionated and estimated by the colorimetric method. Dinitrophenol was added in 2×10^{-4} *M*.

1) No addition of dinitrophenol

	Administered		Remained in intestine			Absorbed	
	Hexose	P	Inorg. P	Org. P (bound hexose)	Free* hexose	Hexose	Per cent
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
Glucose	60.0	0	0	0	3.4	56.6	95.6
Fructose	„	„	„	„	17.5	42.5	70.8
G-1-P	„	10.3	4.8	2.5 (14.5)	3.3	42.2	70.3
G-6-P	58.7	10.0	3.2	2.8 (16.3)	2.1	40·3	68.7
F-1,6-diP	30.4	10.5	3.8	4.4 (12.8)	2.3	15.3	50.3
2) Addition of dinitrophenol							
Glucose	60.0	0	0	0	24.2	35.8	59.7
Fructose	„	„	„	„	34.5	25.5	42.5
G-1-P	„	10.3	3.7	2.1 (12.2)	4.2	43.6	72.7
G-6-P	58.7	10.0	3.4	2.3 (13.5)	3.0	42.2	71.9
F-1,6-diP	30.4	10.5	3.8	4.9 (14.2)	2.6	13.6	44.7

* Reducing power was measured on the Ba-soluble alcohol-insoluble fraction by the method of Hagedorn-Jensen.

Each value is the average of 4 determinations.

just as has been described by Naito (*2*).

The results of the experiments on intestinal phosphatase are given in Table VI. This enzyme appeared to be highly active on G-1-P, while splitting G-6-P in a smaller degree. β-glycerophosphate was also split to a considerable extent.

In Table VII are summarized the velocities of absorption of hexoses and hexose phosphate esters and the effects of dinitrophenol on their absorption.

Although G-6-P and F-1,6-diP were absorbed at the same rate as those obtained by Mathieu (*20*), G-1-P, which he did not examine, was absorbed at a higher rate than G-6-P. Among the esters examined F-1,6-diP appeared to be absorbed at the lowest rate.

Dinitrophenol, which is known to prevent the process of absorption of hexoses from the intestine, has been demonstrated by a number of workers to inhibit markedly and probably specifically the process of phosphorylation.

Dinitrophenol added to the hexose solution, reduced considerably the rate at which hexoses were absorbed and did not afffect in any way the absorption of their esters. The fact that free hexose with and without dinitrophenol were absorbed at different rates must be taken to indicate that a considerable amount of hexose is phosphorylated to be absorbed in the intestinal mucosa. However, it was also found that the hexose with dinitropnenol is absorbed to a considerable extent.

DISCUSSION

Radioautography showed that the main esters accumulated during the absorption of glucose within the rat intestinal mucosa comprised G-1-P, G-6-P and F-1,6-diP.

A primary phosphorylation of free glucose at the aldehyde group have not yet been demonstrated. It is therefore presumable that the primary product of the absorption of glucose may be G-6-P.

The fact that phosphoglucomutase takes part in the mechanism for the absorption of glucose appears to make it reasonable to conclude that G-6-P in the mucosa is converted to G-1-P to some extent in response to the presence of energy rich phosphate compound.

The present experiment has shown that G-1-P is much more sensitive to the intestinal phosphatase than G-6-P, and that G-1-P is absorbed from the intestine a little more quickly than G-6-P. Accordingly, it appears that most of the G-1-P formed in the intestinal mucosa is split off into

sugar and inorganic phosphate to be left there in the form of free glucose. This assumption is supported by the investigation of Hers et Duve (*22*) in which it was demonstrated that there exists no specific glucose-6-phosphatase in the intestine.

However, it was found in this experiment that G-6-P was also split off enzymatically to a small extent by the purified intestinal phosphatase.

A part of the fructose absorbed appeared as G-6-P on the radio-autogram, and the amount in which it appeared was far greater in the animals treated than in the controls. Moreover there appeared one spot, besides another unknown spot, which might be considered to denote F-1-P. (Unfortunately the lack of samples prevented a further test of the fructose esters.)

This findings lead to the assumption that the most part of the F-1-P formed in the intestinal mucosa may be, if not all, transformed into G-6-P. The assumption may be supported by the investigation of Hers *et* Kusaka (*23*) in 1953, who found that F-1-P in the liver is transformed into G-6-P *via* C_3 compounds, F-1,6-diP and F-6-P[8] as intermediates. However, it is possible in actual fact that there may exist in the intestinal mucosa some more complicated phosphorylating-dephosphorylating systrms concerned in the transformation.

It is also conceivable that, although a portion of the F-1-P may be transformed into the G-6-P, the rest of the ester will become free fructose by dephosphorylation. The observation of Kjerulf-Jensen (*24*) that the ketose level in the plasma of the portal vein during the absorption of fructose surpasses that of the arterial blood, supports the above presumption.

Lundsgaard (*25*), in 1939, reported that the glycogen content of the intestinal mucosa is not increased during the absorption of the hexoses—the fact confirmed by Kenmochi (*26*) in this laboratory. The present experiments, however, have shown, that the amount of glycogen increases, and that the level to which it increases seems to be proportional to the velocity with which hexoses are absorbed from the intestine, the report of Naito (*2*), in 1944, being confirmed.

It was found that ATP accelerated the conversion of G-6-P to G-1-P through the activity of phosphoglucomutase by reversing the direction in the reaction.

In 1949, Meyerhof and Green (*27*) demonstrated that an energy-rich compound such as phosphocreatine alters the equilibrium of

8) Fructose-6-phosphate.

phosphorylation and dephosphorylation. The interesting fact that the equilibrium is reversed in the presence of energy rich phosphate, endorses the result of Meyerhof and Green's experiments.

SUMMARY

1. The esters accumulated in the mucosa of the rat small intestine during the absorption of the sugar given were identified as follows by means of paper chromatography combined with radioautography:

a. Glucose: G-1-P, G-6-P and F-1,6-diP.
b. Fructose: G-6-P, F-1,6-diP, F-1-P and an unknown ester.
c. Galactose: could not be decided.

2. G-1-P, G-6-P and F-1,6-diP were also found each in an appreciable amount in the intestinal mucosa in rats which had been starved for 24 to 48 hours.

3. The existence of phosphoglucomutase in the mucosa of rat small intestine was confirmed.

4. ATP accelerated the conversion of G-6-P to G-1-P by reversing the equilibrium.

5. Glycogen content in the mucosa during hexose-absorption increased.

6. The rate at which different sugar phosphates were split by the purified intestinal phosphatase was varied in this decreasing order:

G-1-P>β-glycerophosphate>F-1,6-diP>G-6-P.

7. The amount in which three phosphorylated sugars, G-1-P, G-6-P and F-1,6-diP, were absorbed in a difinite time was varied in the following order:

G-1-P>G-6-P>F-1,6-diP.

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BIOCHEMICAL STUDIES ON SULFATE-REDUCING BACTERIA

VII. PURIFICATION OF THE CYTOCHROME OF SULFATE-REDUCING BACTERIA AND ITS PHYSIOLOGICAL ROLE

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The presence of a cytochrome in sulfate-reducing bacteria in spite of their anaerobic character, suggests that cytochromes play roles as electron carriers not only in aerobes, but also in anaerobes (*1*, *2*). In fact, it is realized that the cytochrome of sulfate-reducing bacteria was reduced with hydrogen or formate, the substrates of the bacteria, and oxidized with sulfate and other hydrogen acceptors. The low standard redoxpotential of the cytochrome does not conflict with the possible rôle as the intermediary carrier in the reduction of sulfate, *etc.* In order to elucidate the role of the cytochrome in the electron transfer, one must at first separate the cytochrome from each enzyme of the respective reactions and then reconstruct the whole enzyme systems with them. In the previous papers (*3*, *4*), the characterization and separation of thiosulfate reductase and hydrogenase was reported. The present paper reports purification of the cytochrome by ion-exchange chromatography and reconstruction of the systems with it for thiosulfate reduction by hydrogenase. The contents of this paper have already been read (*5*) and its preliminary note has been published in the other journal (*6*). Recently, Postgate reported on a similar method of the purification of the cytochrome and its similar effect on the reaction as well as its chemical nature (*7*).

METHODS AND MATERIALS

Materials—Method of cultivation was same as reported previously (*3*). In some

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experiments acetone-dried cells were used.

Cell-free extracts prepared by freezing and thawing were employed in some experiments. Cells of the used strain of this bacterium were easily disrupted by a repetition of freezing and thawing several times. In many experiments, cell suspension in 0.0067 *M* phosphate buffer, pH 7.0, was employed for this procedure. The obtained solution was centrifuged and clear brownish red supernatant was used for experiments.

The samples of diphosphopyridine nucleotide (*8*) and flavin-adenine dinucleotide (*9*) were prepared from bakers' yeast, and that of cytochrome c from heat muscle (*10*). Flavin mononucleotide was a preparation of Wako Pure Chemicals and riboflavin was of Merck Company.

Methods for Measurement of Hydrogen Uptake in Thiosulfate Reduction—They were similar to those reported early (*4*). Warburg manometers were employed. Thiosulfate was added from the side arms to the enzyme solution in the main compartments under the atmosphere of hydrogen. After the measurement finished, the vessels were detached and pieces of filter paper soaked in alkali in the center wells employed for the absorption of gases were taken out at once, and sulfide contents were determined according to the method of St. Lorant. Though values obtained were somewhat lower owing to incomplete absorption, this procedure gave roughly semi-quantitative values for the formed sulfide.

For the quantitative determination of sulfide formed, a hydrogen bubbling method was used (*11*).

Hydrogenase Preparation—The particulate fraction obtained from ground cells with alumina was employed as hydrogenase source (*4*).

Assay methods for hydrogenase, thiosulfate reductase, the cytochrome and the green pigment are the same as the previous paper (*4*). The protein nitrogen was determined by micro-Kjeldahl method for precipitates obtained by addition of trichloroacetic acid.

Iron contents of cytochrome preparations were measured by *o*-phenanthroline method (*12*), which was slightly modified for micro-scale. The cytochrome solution obtained by elution from ion-exchanger with 0.25 *M* ammonia was pipetted into a small porcelain crucible and dried at 110° in an oven. The sample was decomposed to ashes over a small flame. The weight of the sample was calculated from the weights of the crucible before and after the decomposition, and corrected by the weight of iron oxide. Ashes were dissolved in 2 *N* HCl and transferred to a small test tube marked at 2.5 ml. The crucible was washed with 1 per cent hydrazine sulfate, *o*-phenanthroline reagent dissolved in 2 *M* acetate buffer, pH 4.5, 2 *N* ammonia and redistilled water in this sequence, and the washings were added to the tube. The contents of the tube were diluted to 2.5 ml. with redistilled water. After warming the tube to complete color development for 30 minutes at 50° in a water bath, it was cooled and the optical density at 510 mμ was measured by means of Beckman spectrophotometer and compared with standard solution as usual. This assay method gave values within 5 per cent errors.

EXPERIMENTALS AND RESULTS

Purification of Cytochrome of Sulfate-reducing Bacteria—2.7 g. of acetone-dried cells were extracted with 54 ml. of 0.067 *M* phosphate buffer, pH 6.4, for 30 minutes at room temperature, and centrifuged at 16,000 r.p.m. for 15 minutes. Cold acetone was added to the supernatant, and precipitate formed between 33 per cent and 67 per cent was taken in 0.0067 *M* phosphate buffer, pH 7.0, and dialyzed against the same buffer. Most parts of hydrogenase present in the crude extracts could be eliminated by this procedure. The dialyzate was passed through a column 2.5 × 6 cm. of ammonium type of Amberlite IRC 50 which was previously treated after Margoliash (*10*). The red pigment remained at the top of the column and the filtrate was green-red. The column was washed with distilled water and the absorbed substances were eluted successively with 0.1 *M* ammonium acetate, a series of mixtures consisted of various proportions of 0.1 *M* ammonium acetate and 0.25 *M* ammonia, and 0.25 *M* ammonia. The effluent was collected into 19 fractions. The absorption spectra, activities of thiosulfate reductase and proteinous nitrogen were determined. The results of this fractionation are indicated in Fig. 1. Examples of typical absorption spectra are shown in Figs. 2 and 3.

From these figures, it is evident that most parts of proteins including thiosulfate reductase and green pigment ran through the column without absorption. On the other hand, the cytochrome was mainly retained and eluted in two fractions. These are conveniently designated as cytochromes I and II.

The fraction 4 which ran through the column had a typical spectrum of green pigment (*4*), though a little amount of cytochrome was also present. The spectra of the fraction 15, one of cytochrome II, were similar in visible region to those of the cytochrome samples previously obtained by other methods (*4*), but absorption in ultraviolet region was much less than the latter. The reduced form has maxima at 553 (α), 525 (β) and 419 mμ (γ), and the oxidized form at 410 (γ) and 350 mμ (δ). Absorption at 280 mμ is generally accepted to be due to some aromatic amino residues and used for assay of proteins. In the case of the cytochrome, there is no absorption maximum at 280 mμ and optical density was very small. Ratio of optical density at α band to that at 280 mμ of re-chromatographed sample was 3.1, which was 2.6 times as large as that of pure cytochrome c. This indicated that the cytochrome itself had a small amount of amino acid residues having absorption

maximum at 280 mμ, and the sample was considerably pure from other proteins.

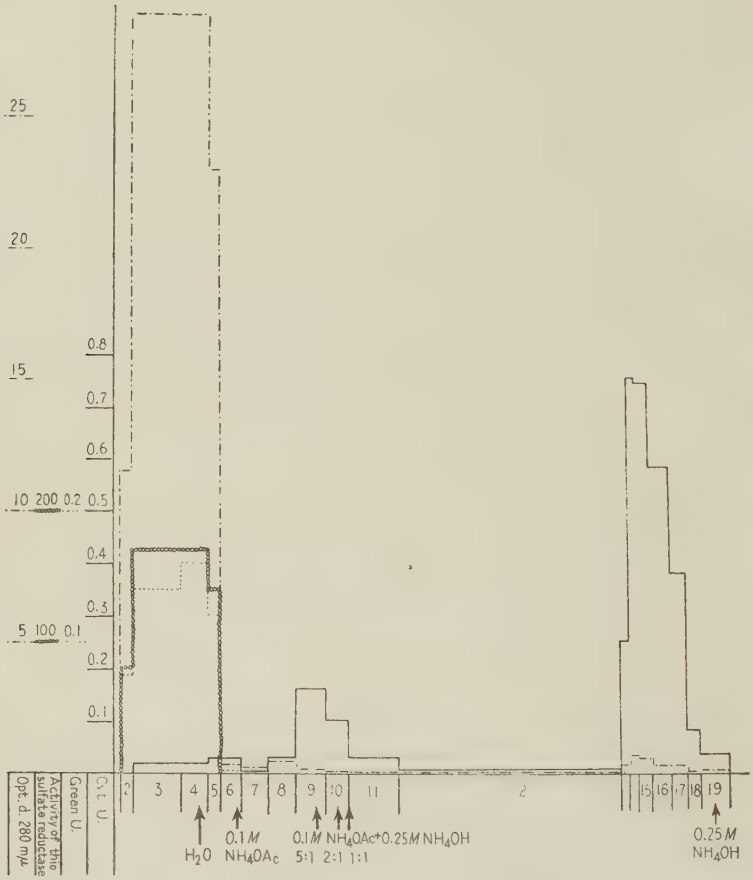

FIG. 1. Ion-exchange chromatogram of extracts of acetone-dried sulfate-reducing bacteria.

See text. Solid line : cytochrome concentration (in units per ml.), dotted line : concentration of green pigment (in units per ml.), large dotted line : activity of thiosulfate reductase (in units per ml.), broken line : optical density at 280 mμ.

The iron contents of the sample were 0.9 per cent by weight and specific extinction coefficient was 4.0 at 553 mμ (α). The results were

in good agreement with those of Postgate (*7, 13*).

Reconstruction of the System for Reduction of Thiosulfate with Hydrogen— Employing cytochrome II and fraction 4 which contained thiosulfate reductase and hydrogenase, reconstruction of system for reduction of

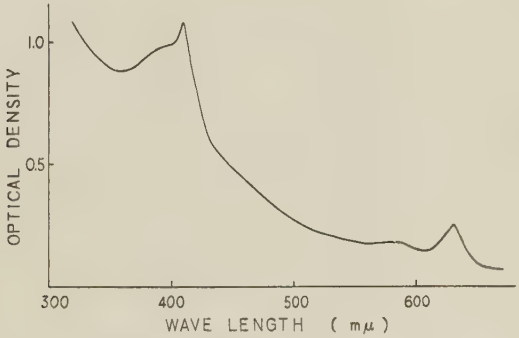

FIG. 2. Absorption spectrum of solution, passed through chromato-column of ion-exchange resin.

Fraction 4, indicated in Fig. 1, was employed. Light path : 1 cm.

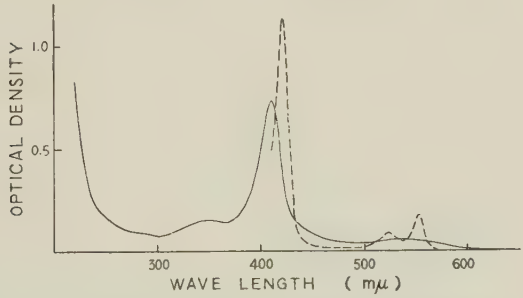

FIG. 3. Absorption spectra of a cytochrome preparation eluted from ion-exchange chromato-column.

Fraction 15, indicated in Fig. 1, was employed. Light path : 1 cm. Solid line : in oxidized state, broken line : in reduced state with sodium dithionite.

thiosulfate with hydrogen was undertaken. Fraction 4 had no activity for the reaction, unless methyl viologen or cytochrome II was added. In the presence of the latters, hydrogen uptake occurred (4.3 μ moles

for 5 μ moles of thiosulfate), as shown in Fig. 4. It was evident that the reduction of thiosulfate to sulfite and hydrogen sulfide occurred. The estimation of the sulfide in alkali in the center wells gave positive

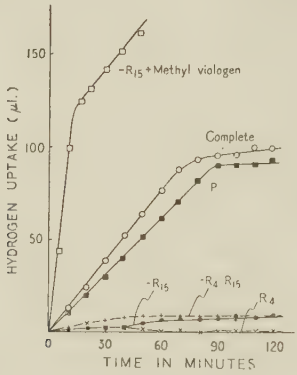

FIG. 4. Reduction of thiosulfate with hydrogen caused by reconstructed systems.

Warburg manometers were employed. Total volume of reaction mixture: 2.25 ml. Temperature; 30°. Atmosphere: hydrogen. Complete system was composed of:

(1) 0.5 ml. of suspension of particulate hydrogenase preparation (p) (80 hydrogenase units), (*4*)

(2) 0.4 ml. of fraction 4 (R4) indicated in Fig. 1 (70 thiosulfate reductase units, almost free from cytochrome),

(3) 0.75 ml. of cytochrome preparation, fraction 15 (R15), indicated in Fig. 1 (0.55 cytochrome units),

(4) 0.2 ml. of 0.2 *M* phosphate buffer, pH 7.0, containing 6 mg. of egg albumin and

(5) 5 μM of sodium thiosulfate.

Center wells contained 0.2 ml. of alkali. Thiosulfate solution was tipped in from side arms and the reaction was started. No hydrogen uptake occurred in the absence of thiosulfate. ○; complete system, □: R15 was omitted and methyl viologen was added, ■: P was omitted, ●: R15 was omitted, ×: R4 was omitted, +: R4 and R15 were omitted.

results only when the hydrogen absorption took place (Table I). To fortify the hydrogenase activity of the fraction 4, hydrogenase preparation was added which had no activity of thiosulfate reductase. Also in that

TABLE I

Sulfide Formation in Thiosulfate Reduction by the Reconstructed System

System	Sulfide (μM)
Complete	4.0
—Sodium thiosulfate	0.22
—Fraction 4	0.26
—Fraction 15	0.59
—Fraction 15, +methyl viologen	5.2
—Hydrogenase preparation	3.4
—Hydrogenase preparation, —Fraction 15	0.0

Hydrogen sulfide was measured which was absorbed by filter paper in alkali in the center wells of Warburg vessels in the course of the experiment, the hydrogen uptakes of which were indicated in Fig. 4. See Methods.

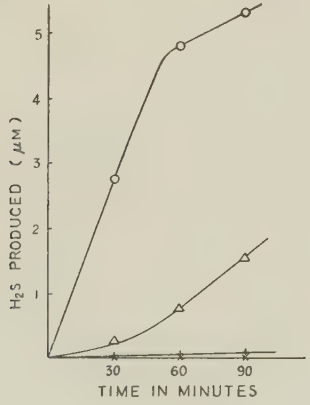

FIG. 5. Evolution of hydrogen sulfide by reduction of thiosulfate with hydrogen.

Reaction mixture consisted of 0.4 ml. of fraction 4, 1 ml. of fraction 16, indicated in Fig. 1., 0.1 ml. of 0.04 *M* sodium thiosulfate and 0.2 ml. of 0.2 *M* phosphate buffer, pH 7.0, 0.4 ml. of hydrogenase preparation and 1.5 mg. of egg albumin. Hydrogen was passed through the reaction mixtures and the hydrogen sulfide formed was determined by the method of St. Lorant. Temperature : 30°, △ : Complete system, ○ : methyl viologen was added instead of fraction 15, × : sodium thiosulfate was omitted. + : Fraction 15 was omitted with and and without fraction 4.

case, hydrogen uptake occurred only in the presence of the cytochrome, and not in its absence.

The necessity of cytochrome or methyl viologen for the reaction could be shown by more accurate estimation of hydrogen sulfide formed by bubbling hydrogen through reaction mixtures. The results are indicated in Fig. 5.

When the amount of cytochrome sample was increased, the rate of reaction was also increased proportionally. This relation was the same for several fractions which contained cytochrome II ; *i.e.* the

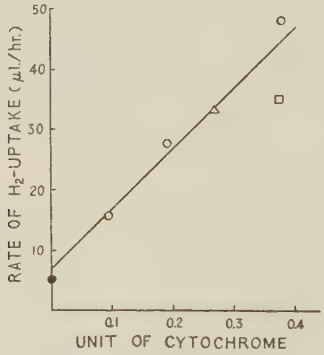

FIG. 6. Relation between concentration of cytochrome and reaction rate of thiosulfate reduction.

Reaction mixtures were composed of : 0.5 ml. of particulate preparation of hydrogenase (*4*) ; 0.4 ml. fraction 3, indicated in Fig. 1 ; 0.2 ml. phosphate buffer, pH 7.0 ; 6 mg. of egg albumin ; 5 μ moles of sodium thiosulfate and cytochrome preparation. Total volume : 2.6 ml. Atmosphere : hydrogen. Temperature : 30°. As cytochrome preparation, utilized respectively ○ : fraction 17, △ : fraction 16, □ : fraction 14, ● : none.

cytochrome concentrations of the samples estimated optically were proportional to their activities as the intermediary carrier (Fig. 6).

Dialysis of the cytochrome sample did not affect the velocity of the hydrogen uptake.

The activity of the cytochrome was the same order as methyl viologen in same moler concentration.

Cytochrome fraction I was reduced with hydrogen in the presence of hydrogenase, but had no activity in the reaction. Cytochrome c,

flavin-adenine dinucleotide, flavin mononucleotide, riboflavin and diphosphopyridine nucleotide could not replace methyl viologen.

DISCUSSION

Observation that methyl viologen enhanced the reduction rate of thiosulfate with hydrogen in the extracts of sulfate-reducing bacteria was considered to indicate the role of methyl viologen as an intermediary electron carrier in the reaction (*4*). This was confirmed by the fact that hydrogenase which reduced methyl viologen with hydrogen, and thiosulfate reductase which oxidized reduced methyl viologen with thiosulfate, were obatined free from each other, and by reconstruction of the system with them. The following reaction scheme was established.

$$H_2 \xrightarrow[\text{hydrogenase}]{} \text{Intermediary carrier} \xrightarrow[\text{thiosulfate reductase}]{} \begin{matrix} S_2O_3^{--} \\ \downarrow \\ SH_2 + SO_3^{--} \end{matrix}$$

The pure cytochrome preparation, obtained by ion exchange chromatography, had the same activity as methyl viologen for the reduction of thiosulfate with hydrogen in the presence of enzymes. The fact that activities of several preparations, obtained from the same chromatographic zone, were proportional to the concentrations of cytochrome, and that the dialyzed preparation had the same activity as the untreated preparation suggested the active principle to be the cytochrome itself.

The cytochrome probably behaved as an intermediary carrier, from the analogy to the role of methyl viologen in the same reaction. But in the crude extracts, the cytochrome which could readily be reduced with hydrogen could not be re-oxidized with thiosulfate. These facts, however, are due to the high standard redox potential of the cytochrome (—0.205 volt (*7*, *13*)), compared to that of system thiosulfate-sulfide and sulfite (—0.423 volt, calculated).

Activity of the crude extracts of cells for reduction of thiosulfate with hydrogen depends on the presence of tle cytochrome, because passage through column of cation-exchanger decreased the activity, and addition of cytochrome recovered the activity of the passed extracts.

In living cells, the cytochrome is considered to play the role of an intermediary carrier in the thiosulfate reduction. In fact, in living cell suspension, its reduction with hydrogen and oxidation with thiosulfate were observed.

It is interesting that one electron carriers such as the cytochrome of

Desulfovibrio or viologen dyes had effect as an intermediary electron carrier, and two electron carriers including diphosphopyridine nucleotide, flavin-adenine dinucleotide, flavin mononucleotide, riboflavin and some synthetic dyes (*3*) had no effect. Only cytochrome c was out of case which we found. Its inability may depend on its too high redox potential.

No action of cytochrome I, the more easily elutable cytochrome fraction, may be due to the fact, that the cytochrome I is the denatured product of the cytochrome.

Though sulfite reductase was extractable (*3*), clear results which indicate the participation of the cytochrome to sulfite-reduction have not been achieved owing to its low activity. Although Postgate showed its slight effect, the cytochrome was often found to be inactive for the crude extracts in our experiments. For the confirmation, purification of enzymes is necessary.

Recent studies dealing with electron transport systems revealed the participation of cytochromes in various reactions which had been assumed not to contain cytochromes as an essential components for a long time. Typical examples are nitrate reducing system in facultative anaerobes (*14*) and photooxidative system in photosynthetic anaerobes (*15*, *16*). Our results presents another example, in which thiosulfate is reduced with hydrogen by two enzyme system mediated by one of cytochromes. It is anticipated that cytochromes will be found to be participate in other anaerobic respirations.

SUMMARY

Cytochrome of sulfate-reducing bacteria, strict anaerobes, was purified by ion-exchange chromatography. Purified cytochrome has absorption maxima at 553, 525 and 419 mμ in its reduced form and 410 and 350 mμ in its oxidized form. Absorption at 280 mμ is very low. Iron contents are determined to be 0.9 per cent. Reconstruction studies with the cytochrome and the extracts deprived of the cytochrome revealed its participation as the intermediary electron carrier between hydrogenase and thiosulfate reductase.

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THE KINETICS AND STOICHIOMETRY OF THE OXIDASE REACTION BY TURNIP PEROXIDASE

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In the oxidase reaction catalyzed by peroxidase, MB and thionine serve as H-acceptor instead of O_2 in the presence of RnH_2 or DHM as H-donor, provided that a suitable amount of H_2O_2 is added (*1*).

$$RnH_2 + MB \xrightarrow[(H_2O_2)]{TP} Rn + MBH_2 \qquad (1)$$

But it is not yet clear whether H_2O_2 acts as a catalytic agent or as a reactant in the oxidase reaction caused by peroxidase.

In the present work, the kinetics and stoichiometry of this MB reducing reaction catalyzed by TP were studied in greater detail.

EXPERIMENTAL

The preparation of TP and RnH_2 was described in the previous paper (*2*). The optical density of the dye was determined by a Beckman spectrophotometer DU type. The decolorizing reactions were measured in the manner described previously (*1*). Nitrogen was de-oxygenated carefully by repeated passage through alkaline solution of pyrogallol and further over heated copper, especially in the determination of the stoichiometry of the reaction.

RESULTS

When the concentration of TP is sufficiently low the initial rate of the MB decolorizing reaction is proportional to the TP concentration (*1*). Fig. 1 shows the pH dependence of the initial rate of peroxidase and MB decolorizing reaction in the presence of RnH_2. These pH

The following abbreviations are used : TP, turnip peroxidase ; RnH_2, triose reductone ; Rn, 2 equivalents oxidized form of RnH_2 ; DHM, dihydroxymaleic acid ; MB, methylene blue ; HRP, horseradish peroxidase.

activity curves are also analogous to that of aerobic oxidation of RnH_2 by TP (*2*). Mn^{++} exhibits an activating effect on the aerobic oxidase activity of peroxidase (*2–9*), but not in the peroxidatic nor MB decolorizing reaction catalyzed by TP in the presence of RnH_2.

The molar ratio of reduced MB to added H_2O_2 varies considerably according to the experimental conditions. It decreases remarkably at high TP concentrations above 0.01 μM at pH 5.30, but is almost constant at pH 2.98 (Fig. 2). pH dependence of the ratio is shown in Fig. 3. In this experiment most suitable TP concentration was used at each pH so as to obtain the highest ratio in each case. At neutral pH no reduc-

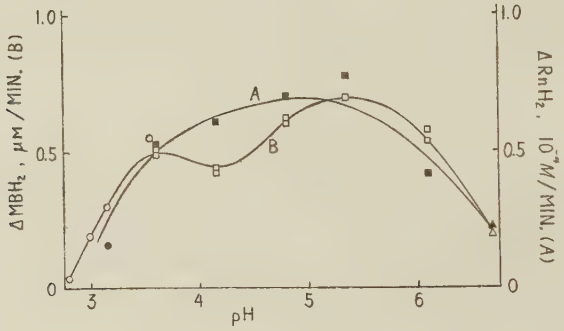

FIG. 1. PH dependence on the initial rate of peroxidase (A) and MB-decolorizing (B) reaction in the citrate (○, ●), acetate (□, ■), and phosphate (△, ▲) solutions under the anaerobic conditions.

A (●, ■, ▲) : 0.4 mM RnH_2, 0.2 mM H_2O_2, 4×10^{-9} *M* TP, room temperature (14°).

B (○, □, △) : 0.4 mM RnH_2, 20 μM H_2O_2, 5 μM MB, 2×10^{-10} *M* TP, room temperature (16°).

tion occurred, but it is of interest that the ratio is almost unity at acidic pH. Although the amount of reduced MB increases with H_2O_2 concentration (*1*), the molar ratio of reduced MB to added H_2O_2 decreases on the contrary. Fig. 4 shows that the ratio increases with the decrease of H_2O_2 concentration and becomes almost unity when extrapolated to zero H_2O_2 concentration. These experimental results indicate that one mole of H_2O_2 is consumed to reduce one mole of MB under the experimental conditions in which H_2O_2 is used effectively for the reduction of MB.

It is important to ascertain whether CO exhibits an inhibitory effect

or not, but no reliable results have so far been obtained. Swedin

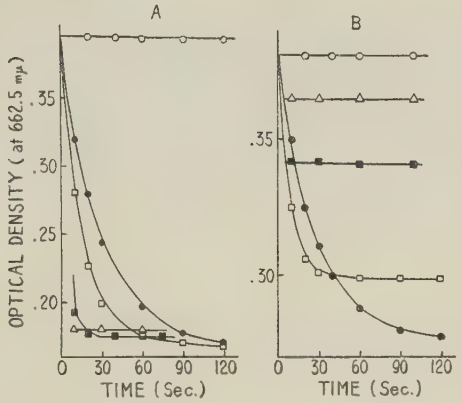

Fig. 2. Effect of the high TP concentration on the reaction curve of MB-decolorization under the anaerobic conditions, room temperature (14°).

A : 0.02 *M* citrate, pH 2.98. Concentrations of TP are : 0 (○), 0.01 μM (●), 0.02 μM (□), 0.1 μM (■), and 1 μM (△).

B : 0.02 *M* acetate, pH 5.35. Concentrations of TP are : 0 (○), 0.004 μM (●), 0.02 μM (□), 0.1 μM (■), and 0.3 μM (△).

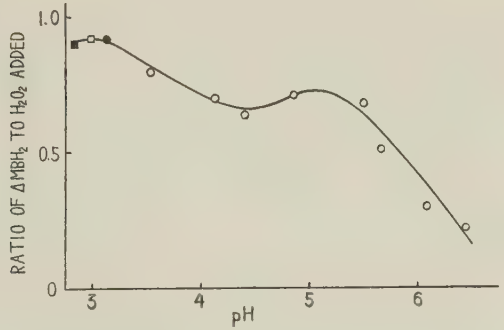

Fig. 3. pH dependence on the ratio of reduced MB to H_2O_2 added under the anaerobic conditions.

1 mM RnH_2, 1 μM H_2O_2, 5 μM MB, 0.02 *M* citrate-phosphate buffer, room temperature (17°). TP concentrations are: 0.01 μM (○), 0.02 μM (●), 0.04 μM (□), and 0.1 μM (■).

and Theorell (*4*) have already reported that CO exhibits an in-

hibitory effect on the aerobic oxidase activity of HRP, and that the effect is removed by light. They also obtained an absorption spectrum of ferroperoxidase-CO compound by bubbling CO through the solution of HRP and DHM. Recently Ray (*10*) studying the direct oxidase reaction of omphalia peroxidase on indoleacetic acid, has proposed that ferroperoxidase may be involved in the reaction. But Chance (*5*) has reported that ferroperoxidase is not involved in the DHM oxidase reaction catalyzed by HRP. In this MB decolorizing reaction only slight inhibition is observed even in the CO saturated solution, as shown in Fig. 5. Moreover, on bubbling CO through the solution of TP

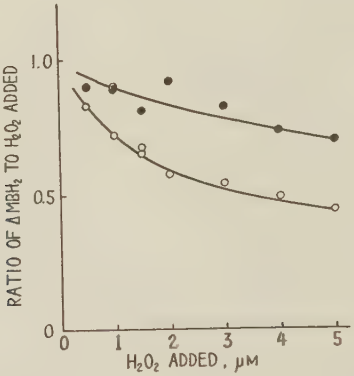

FIG. 4. Effect of H_2O_2 amounts added on the ratio of reduced MB to H_2O_2 added under the anaerobic conditions. 0.8 mM RnH_2, 5 μM MB, room temperature (13°–14°). ●: 0.02 *M* citrate, pH 2.98, 0.05 μM TP. ○: 0.02 *M* acetate, pH 4.78, 0.01 μM TP.

and RnH_2 in the presence or absence of H_2O_2, no spectral change that might be indicative of the formation of ferroperoxidase-CO compound could be observed.

DISCUSSION

According to the view held by Theorell and Swedin (*4*), HRP acts as an aerobic oxidase by means of the shuttling valency change of peroxidase iron. But Chance has reported that the catalyst for the oxidase reaction appears to be a Mn^{++}-activated peroxidase-peroxide complex and much smaller activities are obtained with the peroxide complex alone or with Mn^{++}-activated HRP. Recently Ray (*10*) sug-

gested a free radical mechanism for the direct oxidase reaction of omphalia peroxidase in the presence of indoleacetic acid in which ferrous peroxidase may be involved.

On the other hand excellent results have been demonstrated by George (*11*, *12*) that peroxidase-peroxide complex II is reduced to ferriperoxidase in a one equivalent reduction. Chance (*13*) has also showed that complex I and the donor substance react in a second-order fashion at a speed of roughly 50 times that of the reaction of complex II

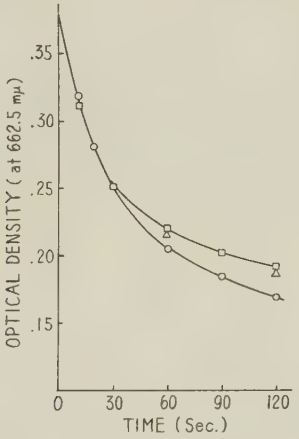

FIG. 5. Effect of CO on the reaction curve of the MB decolorization. 0.001 μM TP, 0.4 mM RnH_2, 20 μM H_2O_2, 5 μM MB, 0.02 *M* acetate, pH 4.69.

○ : in N_2 bubbling solution. □ : in CO bubbling solution.

△ : in CO bubbling solution, light was passed through the solution only when determination was made.

with the donor and established the following peroxidase mechanism.

$$\text{HRP} + \text{H}_2\text{O}_2 \longrightarrow \text{Complex I} \quad (2)$$

$$\text{Complex I} + \text{AH}_2 \longrightarrow \text{Complex II} + \text{AH}\cdot + (\text{H}_2\text{O}) \quad (3)$$

$$\text{Complex II} + \text{AH}_2 \longrightarrow \text{HRP} + \text{AH}\cdot + (\text{H}_2\text{O}) \quad (4)$$

The absence of direct experimental evidence for the formation of free radicals of the donor molecule has remained a weak point in this postulated one step reaction mechanism for peroxidase. However, this peroxidase mechanism seems to have some connection with the oxidase one. The above three reactions lead to the net reaction :

$$2\ AH_2 + H_2O_2 \xrightarrow{HRP} 2\ AH\cdot + 2\ H_2O \qquad (5)$$

If RnH· free radicals which occurred in the peroxidase reaction of RnH_2 react with MB according to the following equation,

$$2\ RnH\cdot + MB \longrightarrow MBH_2 + 2\ Rn \qquad (6)$$

the net reaction in the MB decolorizing reaction by TP in the presence of RnH_2 will be represented as follows,

$$2\ RnH_2 + H_2O_2 + MB \xrightarrow{TP} 2\ Rn + 2\ H_2O + MBH_2 \qquad (1')$$

This equation is in good accord with the stoichiometry obtained experimentally. The molar ratio of reduced MB to added H_2O_2 becomes unity under the experimental conditions in which added H_2O_2 is used most effectively for the reduction of MB, but in other cases the following reactions will also occur, which reduce the ratio below unity.

$$2\ RnH\cdot \longrightarrow RnH_2 + Rn \qquad (7)$$

$$MBH_2 + H_2O_2 \xrightarrow{TP} MB + 2\ H_2O \qquad (8)$$

The rapid formation of free radicals will accelerate the reaction of Equation 7, especially at higher pH (Fig. 2 B). Reduced MB is reoxidized by the same peroxidase system according to Equation 8 when a large amount of H_2O_2 is added or the reaction solution contains only a small amount of RnH_2 which competes with MBH_2 in the reaction of Equation 8. In the aerobic oxidase reaction caused by peroxidase the free radicals which occur in the peroxidase reaction may reduce molecular oxygen to H_2O_2. But the mechanism of interaction between RnH· free radicals and O_2 has not yet been clarified.

The above mechanism is inconsistent with the mechanism in which ferrous peroxidase is involved. The problem of CO inhibition has not completely been resolved, but in the MB decolorizing reaction only a little inhibition is observed and it is not essential to the oxidase activity of peroxidase as Chance (*5*) has already reported in the DHM oxidase reaction by HRP.

The role of Mn^{++} in the oxidase reaction by peroxidase is quite important. Chance (*5*) suggested a Mn^{++}-activated peroxidase-peroxide complex and Kenten (*6*, *7*) emphasized the peroxidatic oxidation of Mn^{++} to Mn^{+++} and concluded that oxidized manganese must react with an intermediate if manganese oxidation plays a part in the reaction. In the RnH_2 oxidase reaction by TP, Mn^{++} also exhibits an

activating effect when O_2 acts as H-acceptor, but no activation is observed when MB acts as H-acceptor. This result suggests that Mn^{++} may have connection only with the interaction between free radicals and O_2.

The mechanism here presented is supported by another experimental evidence that free radicals formed by peroxidase reaction may be separated into two groups, one of which has a reducing power and the other an oxidizing one and the former includes RnH_2, DHM and indoleacetic acid which are substrates of oxidase activity of peroxidase; the results will be given in the following paper.

SUMMARY

In the MB reducing activity of TP in the presence of RnH_2, the molar ratio of reduced MB to added H_2O_2 becomes unity under the experimental conditions in which added H_2O_2 is used most effectively for the reduction of MB. This fact suggests the following mechanism,

$$2\ RnH_2 + H_2O_2 \xrightarrow{TP} 2RnH\cdot + 2\ H_2O \qquad (1)$$

$$2\ RnH\cdot + MB \longrightarrow 2\ Rn + MBH_2 \qquad (2)$$

that is;

$$2\ RnH_2 + H_2O_2 + MB \xrightarrow{TP} 2\ Rn + MBH_2 + 2\ H_2O \qquad (3)$$

The reaction of Equation 1 has been demonstrated by the experiments of George and Chance and the reducing ability of free radical RnH· has also been confirmed.

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STUDIES ON THE METABOLISM OF PARA-AMINOSALICYLIC ACID

II. CHARACTERIZATION OF GLUCURONIDES IN HUMAN URINE AFTER ADMINISTRATION OF PARA-AMINO-SALICYLATE*

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In the previous paper (*1*) one of the authors reported that the administration of *p*-aminosalicylic acid (PAS) to human beings resulted in excretion of unchanged PAS, N-acetyl PAS, *p*-aminosalicyluric acid, a sulfate conjugate and five different glucuronides in the urine. Kawamata and Kashiwagi (*2*) recognized N-acetyl-PAS glucuronide in human urine by means of countercurrent distribution method as well as paper chromatography, but no other glucuronide was detected. Tsukamoto and Yamamoto (*3*) detected PAS glucuronide by paper chromatography in the urine of rabbits after administration of PAS, but the position of conjugation was unclarified.

In this paper, the partial isolation of the five glucuronides in the human urine and the characterization of them by hydrolysis with alkali, acid and glucuronidase will be reported.

EXPERIMENTAL

Preparation of Crude β-glucuronidase—Crude preparation of crude β-glucuronidase was obtained by nearly the same procedure of Karunairatnam and Levvy (*4*). Mouse liver weighing about 1 g. was homogenized in Potter-Elvehjem type homogenizer with a few milliliters of water, 1 ml. of 1 *M* acetate buffer of pH 5.0 was added to the homogenate and, after incubation for 30 minutes at 37°, the mixture was centrifuged for 15 minutes at 1500 g. The supernatant was fractionated between 0.3 and 0.5 saturation of ammonium sulfate. The precipitate was dissolved in a minimum volume of water and dialyzed against water in a cellophane sack for 48 hours at 0 to 5°.

* The subject matter of this paper was presented at a meeting of Kanto Branch of the Biochemical Society of Japan in January, 1957.

Saccharate was used as an inhibitor of β-glucuronidase (*5*). Saccharic acid which was prepared by oxydizing glucose with nitric acid was dissolved in water, adjusted to pH 3.0 with diluted NaOH and placed in a boiling water bath for about 30 minutes for lactone ring formation. The solution was then adjusted to pH 5.0 with potassium carbonate.

Paperchromatography—the method was essentially the same as previously described (*1*).

Chemical Analyses—The quantity of PAS was determined by Bratton-Marshall's method for the determination of sulfonamide. One-tenth ml. of the sample was hydrolysed with 1 *N* KOH, neutralized with HCl and diluted to about 1 ml. The solution as well as the reagents to be used were cooled previously in an ice water bath. To the hydrolyzed sample 0.5 ml. of 0.5 per cent sodium nitrite was added, after 2.5 minutes followed by 0.5 ml. of 0.5 per cent ammonium sulfamate and after 1.5 minutes by 0.5 ml. of 0.1 per cent N-naphthylethylenediamine hydrochloride. The mixture was allowed to react for 20 minutes, then diluted with water to 10 ml., and the optical density at 530 mμ was measured by means of a spectrophotometer. There was a linear relation between the quantity of PAS and the optical density.

Glucuronic acid determinations were done by using Jarrige's method (*6*). One milliliter of 1 per cent naphthoresorcinol in 15 per cent sulfuric acid was added to 1 ml. of the sample and followed by 2 ml. of 30 per cent sulfuric acid. The mixture was placed in a boiling water bath for 30 minutes. After cooling in an ice water bath for 5 minutes, the developed color was determined electrophotometrically at 570 mμ.

RESULTS

In the discussion which follows, it will be helpful to refer to the Paper I (*1*) in which Table I represents the data of the metabolites of PAS. The spots No. 4, 5, 6, 8 and 9 have been proved due to glucuronides. Further evidence has been obtained by treating the sample with β-glucuronidase as follows.

Three grams of PAS sodium were administered to human beings, and the urine excreted for 24 hours was concentrated to about one tenth of the original volume. The salts precipitated after cooling the concentrated urine in a freezing mixture were removed. As shown in Fig. 1 and Table I, the spots No. 4, 5, 6, 8 and 9 disappeared, after incubating the desalted concentrated urine with the crude β-glucuronidase. Moreover, the addition of saccharate inhibited the disappearance of spots No. 6, 8 and 9, although the spots No. 4 and 5 were not detected as before, probably due to the action of deacetylating enzyme in the enzyme preparation as discussed below.

As reported in the foregoing paper (*1*), the spots No. 6 and No. 9 have been presumed to be glucuronides of PAS conjugated with -COOH

and -OH, respectively, and the spots No. 4 and No. 5 to be N-acetylated forms of the both types of glucuronides.

For the identification of the spot No. 6 (R_f=0.28), isolation of the compound was carried out as follows.

After intravenous injection of 5 g. of sodium salt of PAS to a dog weighing about 7 kg., the urine excreted for 24 hours was collected.

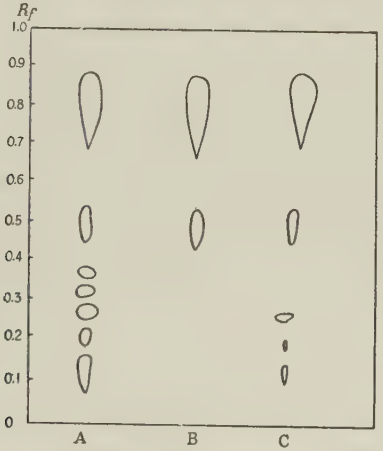

FIG. 1. Schematic paper chromatograms of urine concentrate containing PAS metabolites after hydrolysis with β-glucuronidase. The test system consisted of 0.5 ml. of desalted urine concentrate ; 0.5 ml. of 0.5 *M* acetate buffer, pH 5.0 ; 0.5 ml. of enzyme preparation (omitted in Vessel A) ; and 2.0 ml. of 0.01 *M* saccharate solution (omitted in Vessel B). Final volume ; 3.5 ml. Incubation at 37° with addition of a few drops of toluene as antiseptics. After 20 hours, samples were quantitatively submitted to one-dimensional paper chromatography, employing butanol-acetic acid-water (4:1:5) as the developing solvent. The spots obtained were visualized by spraying with Bratton-Marshall's reagent and Ehrlich's reagent (*1*).

The administration was repeated three times, and the urine corresponding to 15 g. of PAS (about 400 ml.) was condensed *in vacuo* below 50°. As will be reported later (*7*), the dog urine gives only four spots, No. 2, 6, 7 and 8 on paper chromatogram after spraying with Ehrlich's reagent. To destroy the sulfate conjugate (spot No. 7) and the unknown glucuronide (spot No. 8), the concentrated urine was hydrolyzed in 1 *N*

HCl for 20 minutes in a boiling water bath, neutralized with NaOH and the precipitated salt was filtered off. The filtrate was adjusted to pH 3.4 with diluted HCl, and PAS (spot No. 2) was removed by extracting four times with freshly distilled peroxide free ether. The aqueous layer was warmed to drive off the dissolved ether, adjusted to pH 3.4 with acetic acid, and saturated lead acetate solution was added to it until no more precipitate appeared. The precipitate was filtered off. The filtrate was adjusted to pH 8.0 with ammonia water, and basic lead acetate solution was added until no more precipitation took place. The precipitate was collected on a filter, washed with a small quantity of water three times, suspended in a small quantity of water and decomposed with hydrogen sulfide. Precipitated lead sulfide was filtered off. The filtrate, which gave only one spot No. 6 on the paper chromatogram, was passed through a short column of Amberlite IR 50 (H form) and Amberlite IR 45 (OH form) successively, treated with Norit, concentrated *in vacuo* and placed in an ice box. No crystal formation was seen. After the residue was dried *in vacuo* and extracted with anhydrous alcohol, anhydrous ether was added to the extract. Again no crystal was obtained.

The partially purified substance (No. 6) was faint yellow viscous liquid and gave a red color with Bratton-Marshall's reagents, violet color with ferric chloride, a prompt Ehrlich's reaction and a positive naphthoresorcinol test. After hydrolysis in 1 *N* KOH the sample was neutralized with perchloric acid and the precipitate of potassium perchlorate was centrifuged off. The supernatant gave only one spot on paper chromatogram which coincided exactly with that of PAS in its R_f value, color reactions and absorption spectrum. On the other hand the sample was placed in an ice box for about two months at pH 4.0, the glucuronic acid was split off liberated meanwhile and could be detected on paper chromatogram run in collidine saturated with water as a spot with R_f value of 0.03 and in butanol-acetic acid-water (4 : 1 : 5) with R_f value of 0.18, where aniline phthalate in butanol was employed as the coloring agent. Furthermore, quantitative analyses revealed that 0.1 ml. of the sample contained 0.60 μM of PAS and 0.57 μM of glucuronic acid.

These facts confirm the identity of this substance with ester glucuronide of PAS. The isoelectric point was between pH 4 and 5, when estimated by paper electrophoresis.

Spot No. 9 was identified with ether glucuronide of PAS as follows.

Human urine was collected after administration of PAS, condensed

in vacuo, and hydrolyzed in 1 *N* KOH for 20 minutes, at 100°. After neutralyzed with perchloric acid and filtered the filtrate was again hydrolyzed in 1 *N* perchloric acid for 20 minutes at 100°. The hydrolysate, which oave spots of No. 2, 3, 9 and a spot corresponding to *m*-aminophenol on the paper chromatogram, was extracted eight times with about equal volumes of ethyl acetate to remove PAS (No. 2), *p*-aminosalicyluric acid (No. 3) and *m*-aminophenol. After evaporating the dissolved ethyl acetate from the aqueous layer, it was treated with crude glucuronidase as described above and a two dimensional paper chromatogram was developed with *n*-butanol-acetic acid-water (4 : 1 : 5) and methanol-benzene-*n*-butanol-water (2 :1 :1 :1). A distinct spot corresponding to PAS was obtained on the chromatogram. Spectrophotometric examination of the enzymatic hydrolyzate obtained without addition of toluene and diluted with 25 volumes of 0.1 *M* phosphate buffer, pH 7, confirmed again the existence of PAS.

This substance (No. 9) was relatively stable to both acid and alkali, and gave no coloration with ferric chloride. Judging from these facts this substance was identified as ether glucuronide of PAS.

As to spot No. 4 and 5 the negative Bratton-Marshall's reaction and the delayed Ehrlich's reaction indicate that the amino group is acetylated. Absence of spot No. 4 and 5 on the chromatogram of dog urine is in accord with this presumption since doo lacks the ability of acetylating the aromatic amines.

The spot No. 4 developed violet color by spraying with ferric chloride and the eluate of this spot gave a spot coinciding with PAS after hydrolysis with 1 *N* NaOH at 100° for 15 minutes when a chromatogram was run and sprayed with Bratton-Marshall's reagents. There facts indicate that the hydroxyl group remains free and glucuronic acid is attached to the carboxyl radical of N-acetyl-PAS.

The spot No. 5 showed neither ferric chloride reaction nor Bratton-Marshall's reaction, but was recognized by spraying with Ehrlich's reagent although the color developed after a few days. This compound gave naturally a positive reaction with naphthoresorcinol and was decomposed by crude glucuronidase. Although no further investigation was made because of the small quantity it is very likely that this compound is N-acetyl-PAS glucuronide in which the hydroxy radical of PAS is conjugated with glucuronic acid.

To examine the nature of spot No. 8, urine of a dog administered PAS was hydrolyzed with 1 *N* alkali to distruct the spots No. 6 and 7, treated with butanol after adjunsting pH to 3.0 to remove PAS and *m*-

aminophenol, then evaporated to dryness *in vacuo* and the residue was extracted with anhydrous alcohol. This sample gave only spot No. 8. This was hydrolyzed with 1 *N* perchloric acid for 20 minutes at 100°, neutralized with KOH to pH 3.0 and extracted with ether. The extract was evaporated to a small amount of brownish colored oil. This oily substance gave a positive Bratton-Marshall's reaction and a possitive ferric chloride reaction. It is noteworthy that this substance immediately reduced ammoniacal silver nitrate in the cold and darkened in the air within a few days even when kept in an ice box. When run

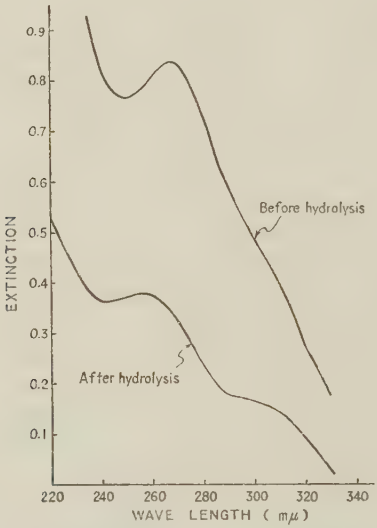

Fig. 2. Ultraviolet absorption curves of the glucuronide corresponding to the spot No. 5 before and after hydrolysis, at pH 7.0.

in butanol-acetic acid-water (4 :1 :5) a spot overlapping with the spot No. 2, was obtained, while when run in methanol-benzene-butanol-water (2 :1 :1 :1) R_f value (0.68) was quite different from that of No. 2 (0.38) as well as those of *m*-aminophenol (0.77) and PAS (0.52). The difference of the R_f values were unequivocally recognized by running the chromatogram of the mixture of this substance, *m*-aminophenol and PAS. The spot of the substance in question gave a violet color as intensive as that of PAS by spraying with Bratton-Marshall's reagents, but the optimum acidity for developing the color was shifted

to more acid side. Ultraviolet absorption curves at 7.0 are shown in Fig. 2.

DISCUSSION

Karunairatnam and Levvy (*4*) reported that saccharate, especially its 1,4-lactone form, inhibited mouse liver glucuronidase, and in addition Levvy and Worgan (*8*) showed that it inhibited also the activity of the β-glucuronidase preparation to hydrolyze ester glucuronide. In this experiment the spot No. 6, 8 and 9 remained when the action of the enzyme was inhibited by addition of saccharate, although the spot No. 9 diminished in size. These results supported the presumption that the three spots are due to glucuronides. The enzyme preparation employed in this experiment was by no means a pure β-glucuronidase, but possibly contained several other hydrolyzing enzymes. That the spots No. 4 and 5 were decomposed by the enzyme even in the presence of the inhibitor might be explained by existence of deacetylating enzyme in the preparation.

Theoretically possible four glucuronides, namely, ester and ether-glucurronides of PAS and N-acetylated PAS, were all demonstrated by the experiments presented here, though not yet completely decided. Tsukamoto and Yamamoto (*3*) detected a glucuronide in rabbit urine after administration of PAS, which gave a spot with R_f value of 0.28 when run in butanol-acetic acid-water (4 :1 :5). This substance is in good agreement with ester-glucuronide of PAS described above. Kawamata and Kashiwagi (*2*) reported the presence of "N-acetyl-PAS glucuronide" as the metabolite of PAS in human urine. Judging from R_f value (0.22 in butanol-acetic acid-water, 4 :1 :2), however, this substance seems to be the glucuronide corresponding to the spot No. 8, although some discrepancies are present in color reactions. Later, Kawamata and Hiratani (*9*) described a glucuronide in rat urine after the administration of PAS, whose R_f value was 0.30 in butanol-acetic acid-water (4 :1 :2), and considered it to be identical with "N-acetyl-PAS glucuronide" in human urine. This substance is probably identical with ester glucuronide of PAS.

Since no one has succeeded in isolating either glucuronide of PAS or of salicylic acid, attempts were made to obtain the pure glucuronides in crystal, but ended in failure. So, the final conclusion about the nature of the glucuronides should be deferred.

The nature of the spot No. 8 has remained unclarified. It seems to

be an ether glucuronide with a free amino group and a free hydroxy group considering from its behavior against alkali treatment and color reactions. The phenolic substance obtained by hydrolysis has been proved to be different from PAS or *m*-aminophenol in its R_f values on paper chromatograms and by ultraviolet absorption spectra. Ultraviolet absorption curve of PAS changed with pH, while that of the hydrolysed product was not. The experimental results suggest that this substance is an aminopolyphenol. The reducing capacity demonstrated by ammoniacal silver test supports this presumption. From these experimental results, the glucuronide corresponding to the spot No. 8 seems to be a glucuronide of an oxidation product of PAS, one of its hydroxyl groups being conjugated with glucuronic acid and the other being free to react with ferric chloride.

Quick (*10*) reported the formation of diglucuronide of *p*-hydroxybenzoic acid. Very recently Bushby and Woiwod reported the occurrence of N-glucuronide of 4,4′-diaminodiphenylsulphone in rabbit urine (*11*). The analogous compound of PAS could not be detected in the present study, though the occurrence of it may not be denied.

SUMMARY

The occurrence of five different glucuronides, ester glucuronide and ether-glucuronide of PAS, the both types of glucuronides of N-acetyl PAS, and presumably an ether glucuronide of oxidation product of PAS, in human urine after ingestion of PAS were demonstrated by paper chromatography in combination with enzymatic hydrolysis.

The authors expresse their thanks to Prof. Yoshikawa for his constant leading and are indebted to Mr. Horie for the use of paper electrophoresis. This work was aided by a Grant from the Scientific Research Fund of the Ministry of Education.

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PHYSICOCHEMICAL STUDIES ON TAKA-AMYLASE A

I. SIZE AND SHAPE DETERMINATION BY THE MEASUREMENT OF SEDIMENTATION CONSTANT, DIFFUSION COEFFICIENT, AND VISCOSITY

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(Received for publication, March 11, 1957)

Taka-amylase A was recently crystallised by Akabori and his collaborators (*1*) from the preparation which was extracted and purified from Taka-diastase. It is generally accepted that the reactivity of protein, especially that of the enzyme, might be affected not only by the chemical constitution (sequence of amino acids) but also by the shape of the molecule as a whole. In this point of view, the investigation of the correlation of the chemical reactivity and the molecular shape would be of the utmost importance. In order to proceed the investigation along this line, we have studied at first the physicochemical characteristics of this enzyme. In the present paper we will describe the results on the determination of molecular weight, size and shape by the measurement of sedimentation constant, diffusion coefficient and viscosity of the solution. We have determined previously the molecular weight of this enzyme by surface chemical technique and was found to be 52,300 (*2*). It was estimated the minimum molecular weight to be 54,000 from the composition of constituent amino acids (*3*) and 52,500 by the quantitative determination of end groups (*4*). The values of the molecular weight of Taka-amylase A found in the investigation by the combination of sedimentation-diffusion, sedimentation-viscosity and diffusion-viscosity were 51,000 if we assume the molecule of a prolate ellipsoid of revolution of axial ratio of 4.00 without or with scarce hydration, and in good agreement with previous data.

EXPERIMENTAL

Material and Solution—The sample of Taka-amylase A used in the present investigation was the one supplied by Prof. Akabori which was crystallised by his method (*1*) and recrystallised three times and was electrophoretically homogeneous.

The sample was dissolved in buffer solution of sodium acetate at pH 4.8, of which ionic strength was 0.2. As it is known that the effect of the charge of protein on its sedimentation constant and diffusion coefficient might be reduced in the solvent containing salt whose ionic strength is more than 0.1, the ionic strength of the solvent was kept to 0.2 in the present investigation. Prior to the diffusion experiment, the protein solution was dialysed against to the acetate buffer solution of the same pH for 72 hours at 0°. The dialysate was used as the solvent of diffusion experiment because it is necessary to keep the concentration of the solutes other than the protein identical in concentration in the solution and in the solvent.

The nitrogen content in the solution examined was determined by the micro-Kjeldahl method from which the amount of protein was estimated assuming the nitrogen content of Taka-amylase A to be 15 per cent (*1*).

Sedimentation—Sedimentation measurements were made with the Spinco Model E ultracentrifuge. The runs were carried out at 59,780 r.p.m. (*i.e.* $259{,}700\times g$) at room temperature (about 20°). The average value of the initial and final temperature of the rotor was used for later calculation as an experimental temperature.

The sedimentation constants were calculated by the following formula : $s_t=2(x_2-x_1)/(x_1+x_2)\omega^2(t_2-t_1)$, where x_1 and x_2 are the distances of boundary from the axis of revolution at the time t_1 and t_2 respectively, and ω is the angular velocity. The sedimentation constants were corrected to 20° in water in the usual manner. The sedimentation constant in water at 20°, $s_{20,w}$, is given by

$$s_{20,w}=s_t\frac{\eta_t}{\eta^0_t}\frac{\eta^0_t}{\eta^0_{20}}\frac{1-\bar{v}_{20}\rho^0_{20}}{1-\bar{v}_t\rho_t}$$

where η_t is the viscosity of the solvent at $t°$, $\eta°_{20}$, $\eta°_t$ the viscosities of water at 20° and $t°$ $\bar{v}_{20}$, $\bar{v}_t$ the partial specific volumes of the protein in solution at 20° and $t°$, $\rho°_{20}$, ρ_t, the density of water at 20° and that of the solvent at $t°$ respectively.

Diffusion—Diffusion was studied by schlieren cylindrical lens method using the cell of Neurath type. Temperature of the thermostat was regulated within ±0.01° after the technique of Moriguchi (*5*) and measurements were carried out at 20°.

The diffusion coefficient was calculated by three different methods, namely, 1) the method of inflexion point, 2) the method of successive analysis and 3) the area method (*6*). The diffusion coefficient at 20° in water, $D_{20,w}$, was calculated from the observed diffusion coefficient, D_t, using the following formula,

$$D_{20,w}=D_t\frac{\eta_t}{\eta^0_t}\frac{\eta^0_t}{\eta^0_{20}}\frac{293}{273+t}$$

Viscosity—Viscosity wss measured at 20±0.05° by the capillary viscometer of Ostwald type of which flow time is 120 seconds.

Partial Specific Volume—The density of the solution and of the solvent was measured at 20±0.05° using the pycnometer of Ostwald type of the capacity of about 3 ml. The apparent partial specific volume $\bar{v}_a$ was calculated by the following formula,

$$\bar{v}_a=\frac{1}{\rho_0}\left(1-\frac{\rho-\rho_0}{w}\right) \quad ,$$

where ρ is the density of the solution, ρ_0 that of the solvent, and w the protein concentration in g./ml.

RESULTS

Sedimentation Constant—A part of photographs of sedimentation diagrams is shown in Fig. 1. The sedimentation constants observed are

FIG. 1. Sedimentation diagrams of 0.94% Taka-Amylase A solution measured at 21° (at pH 4.8 in 0.2 *M* acetate buffer).

Photographs were taken at intervals of 32 minutes at a rotor speed of 59,780 r.p.m.

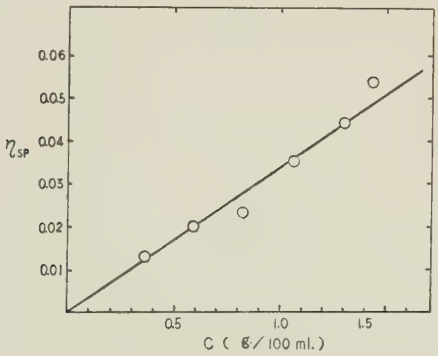

FIG. 2. Plots of specific viscosity *versus* concentration for Taka-amylase A solution measured at 20° (at pH 4.8 in 0.2 *M* acetate buffer).

listed in Table I. They were independent of concentration in the concentration range investigated. The average value of the sedimentation constants at 20° in water was found to be 4.67±0.03 *svedberg*.

Diffusion Coefficient—Diffusion coefficient was calculated from the data obtained in the time interval in which a straight line passing through the origin is obtained if μ^2 (μ is the abscissa of inflexion point) was plotted

against time, because the concentration gradient curve deviates from the Gaussian curve if time has passed more than three hours after making the boundary for diffusion between the solution and the solvent. The diffusion coefficient were evaluated by three different methods as mentioned above. The values of them agreed well with each other. The results of the diffusion experiments are listed in Table II. As shown in Table II, the concentration dependence of diffusion coefficient was not detected with this protein. The diffusion coefficient of Taka-amylase A at 20° in water was evaluated to be $7.37 \pm 0.03 \times 10^{-7}$ cm.²/second by averaging these values.

Viscosity—If the specific viscosity is plotted against the protein concentration, a straight line passing through the origin is obtained as shown

TABLE I

Sedimentation Constant of Taka-Amylase A

The measurements were carried out at pH 4.8 in 0.2 *M* acetate buffer at room temperature (about 20°). Two observations were made for each protein concentration. (1) and (2) are the number of observations.

Protein concentration (g./100 ml.)	$s_{20,w} \times 10^{13}$ (1)	(2)	Mean
0.24	4.82	4.54	4.68
0.47	4.80	4.74	4.77
0.71	4.66	4.55	4.61
0.94	4.79	4.48	4.64
			4.67 ± 0.03

in Fig. 2. In the concentration range where the diffusion and sedimentation measurements were carried out, specific viscosity of Taka-amylase solution changed linearly against concentration, so that the mutual interaction among the protein molecules might be neglected. Accordingly, the reduced viscosity η_{sp}/c is independent of the concentration and we obtained the intrinsic viscosity from the slope of the line in Fig. 2. By dividing this value with partial specific volume, the volume fraction intrinsic viscosity $\nu = 100[\eta]/\bar{v}$ was obtained as 4.86.

Partial Specific Volume—The partial specific volume of Taka-amylase A was determined experimentally. The results are shown in Table III. The experimental value of $\bar{v}$ was 0.700, whereas the partial specific volume

calculated from the specific volumes of constitutional amino acids was 0.73. There was distinct discrepancy between these values. The reason will be discussed in a later paper.

Frictional Ratio and Axial Ratio—The frictional ratio of the molecule, f/f_0 was calculated by the following formula,

TABLE II

Diffusion Coefficient of Taka-Amylase A

The measurements were carried out at pH 4.8 in 0.2 *M* acetate buffer at 20°. D_μ, D_x and D_A are the values calculated by the method of inflexion point, the method of successive analysis and the area method, respectively. D_{mean} is the mean value of them.

Protein concentration (*g./100 ml.*)	$D_{20,w} \times 10^7$ *cm.²/sec.*			
	D_μ	D_x	D_A	D_{mean}
0.25	7.29	7.26	7.44	7.33
0.41	7.38	7.25	7.52	7.38
0.80	7.42	7.51	7.41	7.45
1.04	7.53	7.41	6.98	7.31
				7.37±0.03

TABLE III

Partial Specific Volume of Taka-Amylase A

The measurements were carried out at pH 4.8 in 0.2 *M* acetate buffer at 20°.

Protein concentration (*g./100 ml.*)	Density of solvent (*g./ml.*)	Density of solution (*g./ml.*)	Apparent partial specific volume (*ml./g.*)
0.675	1.0049	1.0069	0.700
1.35	1.0049	1.0089	0.700
2.03	1.0049	1.0109	0.700
		Mean	0.700

$$\frac{f}{f_0} = \frac{1}{\eta}\left(\frac{RT}{DN}\right)^{2/3} \cdot \left(\frac{1-\bar{v}\rho_0}{162\pi^2\bar{v}s}\right)^{1/3}$$

introducing the observed values of s, D and $\bar{v}$. f/f_0 was found to be 1.19. The axial ratio of the molecule was calculated according to the procedure of Oncley (*7*) and Perrin (*8*), assuming the amount of

hydration and the molecular shape. The results are shown in Table IV.

On the other hand, the axial ratio of the molecule can be calculated from the volume farction intrinsic viscosity following the Simha's

TABLE IV

Axial Ratio and Size of Taka-Amylase A

Data calculated from $f/f_0=1.19$ which was determined by sedimentation constant and diffusion coefficient measurements. 2a is the length of the axis of revolution, and 2b that of the axis perpendicular to it.

Molecular shape	Hydration (%)	a/b or b/a	2b (Å)	2a (Å)
Prolate ellipsoid a>b	none	4.17	30	125
	30	2.09	43	90
Oblate ellipsoid b<a	none	4.42	80	18
	30	2.13	70	33
Sphere a=b	48	1.00	58	

TABLE V

Axial Ratio and Frictional Ratio of Taka-Amylase A

Data calculated from intrinsic viscosity.

Molecular shape	Hydration (%)	a/b or b/a	f/f_0
Prolate ellipsoid a>b	none	4.17	1.19
	30	2.64	1.23
Oblate ellipsoid b<a	none	5.23	1.24
	30	2.95	1.24
Sphere a=b	66	1.00	1.25

treatment (*9*). The corresponding frictional ratios was calculated by Perrin's formula (*8*). If the hydration of the molecules was taken into consideration in the calculation of the axial ratio and frictional ratio of the molecule, the ratios might be calculated by the procedure

based on Oncley's method (7). In this case, it is necessary to assume the amount of hydrating water appropriately. It is generally accepted that a gram of protein contains 0.2–0.5 g. of hydration water in aqueous solution. Hence, we calculated the ratios assuming that the protein contains 30 per cent hydration water. However, if we assume the molecule to be a sphere, the amount of hydration is determined inevitably. The results are shown in Table V.

Molecular Weight—The molecular weight was calculated by introducing the observed sedimentation constant, s, diffusion coefficient, D, and partial specific volume, $\bar{v}$ into the following well-known equation,

$$M_{s\text{-}D}=\frac{RTs}{D(1-\bar{v}\rho_0)}$$

Table VI

Molecular Weight of Taka-Amylase A

$M_{s\text{-}\nu}$ is the molecular weight calculated from sedimentation and viscosity data and $M_{D\text{-}\nu}$ that from diffusion and viscosity data.

Molecular shape	Hydration (%)	f/f_0	$M_{s\text{-}\nu}$	$M_{D\text{-}\nu}$
Prolate ellipsoid	none	1.19	51,000	51,000
	30	1.23	54,000	47,000
Oblate ellipsoid	none	1.24	54,000	45,000
	30	1.24	54,000	45,000
Sphere	66	1.25	55,000	44,000

The molecular weight thus evaluated is 51,000, which is in good agreement with other previous data.

The molecular weights can be calculated by the combination of f/f_0 and s, or f/f_0 and D, respectively, by the following formulas,

$$M_{s\text{-}\nu}=\{162\pi^2\eta^3N^2\bar{v}\,(f/f_0)^3\,[s/(1-\bar{v}\rho_0)]^3\}^{1/2}$$

$$M_{D\text{-}\nu}=\frac{R^3T^3}{162\pi^2N^2\eta^3}\,\frac{1}{(f/f_0)^3}\,\frac{1}{\bar{v}D^3}$$

The molecular weights, $M_{s\text{-}\nu}$ and $M_{D\text{-}\nu}$ are listed in Table VI, which are corresponding to various f/f_0 values obtained from volume fraction intrinsic viscosity assuming the molecular shape and the amount of hydration.

The lengths of the major and minor axes were calculated from the axial ratio obtained by the measurements of sedimentation and dif-

fusion assuming the molecular weight of 51,000. The results are shown in Table IV.

DISCUSSION

As the molecular weight may be determined from the sedimentation constant and diffusion coefficient using Svedberg's equation without any assumption concerning the molecular shape and the amount of hydration, it is the most reliable value of the molecular weight compared with the values of molecular weight determined from sedimentation and viscosity and from diffusion and viscosity measurements. The molecular weight of Taka-amylase A determined from s and D was 51,000. By comparing this value with those obtained from s and ν, and D and ν in Table VI, we find that the values of the molecular weight are all in good agreement with each other, if we assume the molecule to be a prolate ellipsoid and unhydrated. Otherwise, $M_{s\text{-}\nu}$ and $M_{D\text{-}\nu}$ deviates fairly from $M_{s\text{-}D}$. On the other hand, the molecular weight of 51,000 agrees well with those determined previously by the surface chemical method and by the analysis of the constitutional amino acids.

The frictional ratio of the molecule determined from s and D is 1.19. This is in good agreement with the frictional ratio determined from ν as shown in Table V, if the molecule is a prolate ellipsoid without hydration. As seen from these results, the identical value may be attained by various methods, if we assume the molecular shape as a prolate ellipsoid, rather than as an oblate ellipsoid, especially if the molecule is not hydrated. Although the non-hydration of the protein molecule in aqueous solution is somewhat incomprehensible, the amount of hydration water in Taka-amylase A molecule would be considerably little.

The consideration on the shape and the hydration mentioned above are due to the treatment based on Simha and Perrin's theories assuming the protein molecule as a rigid particle. It may be concluded that Taka-amylase A molecule should be of a prolate ellipsoid according to the recent theory developed by Scheraga and Mandelkern (*10*) concerning the configuration of protein. The details of the shape and the rigidity of the Taka-amylase molecule will be discussed in a later paper.

If the molecular weight is 51,000 and the frictional ratio 1.19, and if the molecular shape is a prolate ellipsoid of axial ratio of 4.17, the length of the major axis would be 125 Å and that of the minor axis 30 Å, respectively. On the other hand we measured the thickness of

the adsorbed film of Taka-amylase A by the optical method and found 31 Å.[1] This agrees very well with the minor axis of the prolate ellipsoid model of Taka-amylase A, suggesting that the molecules are lying flat in the adsorbed film on solid. From these results, we conclude that the molecule of Taka-amylase A may be a prolate ellipsoid in shape and its hydration would be scanty.

SUMMARY

1. The molecular weight and molecular shape of Taka-amylase A were studied by the measurements of sedimentation constant, diffusion coefficient and viscosity.

2. If we assume the molecule of a prolate ellipsoid of revolution of axial ratio 4.00 without or with scarce hydration, the molecular weight would be 51,000, which is in good agreement with previous data. The length of the major axis of the molecule is 125 Å and that of the minor axis 30 Å, respectively.

3. The partial specific volume of Taka-amylase A is 0.700 and less than the calculated value from the specific volumes of constitutional amino acids.

In conclusion, the authors express their hearty thanks to Prof. S. Akabori and Mr. T. Ikenaka, of the Faculty of Science, Osaka University who supplied us the valuable sample of Taka-amylase and the authors are also indebted Prof. K. Fukai of the Institute for Microbiological Desease, Osaka University for his kind guidance for taking the photographs of sedimentation diagram.

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STUDIES ON CYTOCHROME C

I. PURIFICATION PROCEDURES AND PROTEINASE DIGESTION OF BAKER'S YEAST CYTOCHROME C

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Recently, we succeeded in the preparation of cyrstalline cytochrome c from various sources (*1–4*), *i.e.*, baker's yeast (*1*) and heart muscles of bovine and pig (*2*). Margoliash (*5*) has reported that native horse-heart cytochrome c shows only two chromatographic species on a cation exchanger, reduced and oxidized cytochrome c, the other fractions being artifacts of the preparations, and trichloroacetic acid (TCA) modifies a variable proportion of any sample of cytochrome c into a form indistinguishable spectrophotometrically from the normal cytochrome c. Henderson and Rawlinson (*6*) have reported that the cytochrome c treated with TCA is slightly higher in the oxidation-reduction potential than the resin-purified cytochrome c, owing to the presence of modified cytochrome c. Though our procedure for preparation of yeast cytochrome c never requires TCA, the cytochrome c shows several kinds of chromatographic species even if the chromatography is carried out with its oxidized form.

On the other hand, some enzymes can be protected from denaturation in the presence of their substrates or co-enzymes (*7–9*). suggesting a certain change of inner structure by the formation of enzyme-substrate (-coenzyme) complex. However, addition of fairly large molecules such as their substrates or co-enzymes complicates the explanation, since there are many substances besides the substrates and co-enzymes, which are able to neutralize denaturation of proteins (*10–16*), and the protection of enzyme denaturation by the substrate and co-enzyme has been rather exceptionally demonstrated. The adoption of oxidized and reduced cytochrome c for this purpose may eliminate such a confusion, though taking it into consideration that it is not an enzyme.

The present paper describes effect of the purification conditions on chromatographic species of yeast cytochrome c and differences in digestion of oxidized and reduced cytochrome c by bacterial proteinase.

EXPERIMENTAL

Preparation of Crystalline Cytochrome c from Baker's Yeast—This was carried out according to the previous method (*1*) as shown schematically in Scheme 1.

SCHEME 1

Pressed Baker's Yeast (6 kg.)
↓ kneaded and liquified with 600 ml. of ethylacetate, 6 litre of tap water added, concd. NH_4OH added to pH 8–9, and immediately centrifuged
Washed, Autolyzed Cells (*ca.* 3.4 kg.)
↓ 5.2 litre of tap water added, satd. $(NH_4)_2SO_4$ added to 10% saturation, pH adjusted to 7.5 with concd. NH_4OH, allowed to stand for three hours in a refrigerator, and centrifuged by Sharpless centrifuge

Supernatant Solution | Debris
Debris ↓ re-extracted on a half-scale as above
Debris → Supernatant Solution

Supernatant Solution + Supernatant Solution
↓
Combined Supernatant Solution
↓ dialysed against flowing tap water in a refrigerator for 1–2 days
Dialysate
↓ passed through the XE-64 or CS-101 resin column
Charged Column
↓ eluted with 20%-satd. $(NH_4)_2SO_4$ soln., pH 9.0
Eluate (cytochrome c fractions)
↓ solid $(NH_4)_2SO_4$ added to 90% saturation, immediately centrifuged at 14,000 × *g* for 15 mins.
Supernatant Solution
↓ dialyzed against flowing tap water for 1–2 days in a refrigerator
Dialysate
↓ oxidized with a min. amount of $K_3Fe(CN)_6$, and passed through the XE-64 column
Charged Column
↓ developed with $(NH_4)_2HPO_4$-H_3PO_4 buffer, pH 7.0 (0.3 *M* as NH_4 ion)
Cytochrome c Fractions
↓ concentrated by the same method as the first XE-64 or CS-101 chromatography
Concentrated Cytochrome c Solution
↓ crystallized
Cytochrome c Crystals

Preparation of Oxidized and Reduced Cytochrome c—Cytochrome c solution was completely oxidized and reduced by addition of a minimum amount of potassium ferricyanide or aeration at pH 2.5 and by sodium dithionite or hydrogen in the presence of palladium asbestos, respectively, and then dialysed against $M/50$ Na_2HPO_2 solution overnight in a refrigerator.

Treatment of Cytochrome c with TCA—To the cytochrome c solution saturated with ammonium sulfate 1/20 volumes of 3 *M* TCA neutralized with sodium hydroxide was added and the cytochrome c precipitated was collected on a glass filter. The precipitate was dissolved in $M/50$ Na_2HPO_4 and dialysed against $M/50$ Na_2HPO_4 overnight in a refrigerator. The temperature and the duration of treatment with TCA will be described in each case.

Resin Chromatography of Cytochrome c—Ion exchanger, Amberlite XE-64 (100–150 mesh), was activated as in the case of preparation of crystalline cytochrome c (*1*) and then buffered at pH 7.0 using $(NH_4)_2HPO_4$–H_3PO_4 buffer (0.3 *M* as NH_4 ion). The oxidized cytochrome c was passed through the exchanger column and developed with the same buffer.

Preparation of Crystalline Cytochrome c from Bovine Heart Muscle—Refer our previous report (*2*).

Preparation of Crystalline Cytochrome c from Horse Heart Muscle—This was prepared and crystallized by the same method as from bovines (*2*).

Preparation of Yeast Lactic Dehydrogenase—This enzyme was prepared from the pressed baker's yeast by the method of Bach, Dixon, and Zerfas (*9*).

Preparation of Crystalline Bacterial Proteinase—This was prepared from *Bacillus subtilis* (*17*).

Digestion of Cytochrome c by Bacterial Proteinase—To 20 ml. of the cytochrome c solution adjusted to about 0.1 per cent based on the optical density, 10 ml. of 0.015 per cent bacterial proteinase dissolved in $M/50$ phosphate buffer (pH 7.5) was added and incubated at the desired temperature. From the mixture, 3.0 ml. of the sample was periodically pipetted out and immediately poured into 2.0 ml. of 1.2 *M* TCA. After half an hour the mixture was filtered and optical density of the filtrate at 280 mμ was measured by a Shimadzu spectrophotometer, the value of which was marked as $(OD)_s$. By the same method, $(OD)_n$ was obtained in which 2.0 ml. of cytochrome c and 2.0 ml. of TCA were mixed, then 1.0 ml. of bacterial proteinase was added, and $(OD)_d$ in which the cytochrome c solution was completely denatured by heating in a boiling water bath for twenty minutes at approximately pH 1.0, after adjustment of pH and its volume completely digested by the proteinase solution and mixed with TCA. Percentage digestion of cytochrome c is definable from the following equation :

$$\text{Digestion Per Cent} = \frac{(OD)_s - (OD)_n}{(OD)_d - (OD)_n} \times 100$$

Determination of Enzymically Active Cytochrome c—To 2.0 ml. of cytochrome c solution 1.0 ml. of 0.5 *M* lactate in $M/15$ phosphate buffer (pH 6.0) and 0.3 ml. of diluted solution of a highly purified yeast lactic dehydrogenase were added and active cytochrome c was determined by the increment of the optical density at 550 mμ, as shown in Fig. 1.

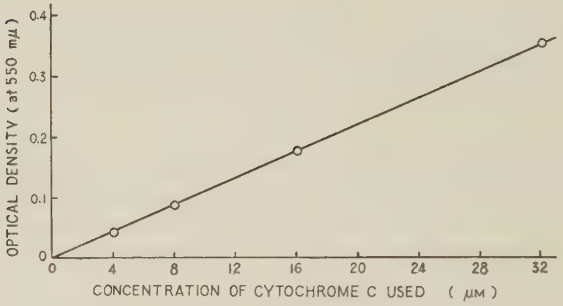


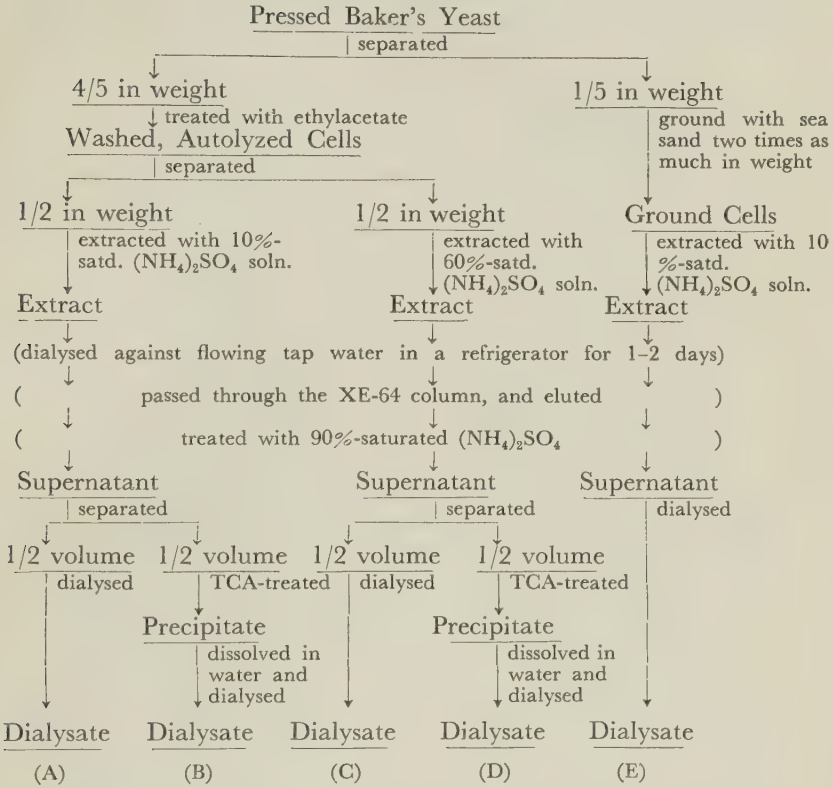
FIG. 1. Standard curve of reducibility of yeast cytochrome c by yeast lactic dehydrogenase.

RESULTS

Effect of Extraction and Purification Procedures of Yeast Cytochrome c on Its Chromatographic Species—It has been reported (*18*) that the cytochrome c purified from baker's yeast has various kinds of chromatographic species in oxidized form and that each of them has a biological activity of the same extent when examined with " Green brei " (*19*). Here, the cytochrome c was prepared by various procedures as shown in Scheme 2 and compared in respect to chromatographic species. The cytochrome c not treated with TCA had a main peak at (IV) fraction as shwon by A, C, and E in Fig. 2, while that prepared with TCA had a main peak at (I) fraction. Each fraction from (I) to (IV) separated from each other had unchanged chromatographic species in re-chromatography, for example as shown in A of Fig. 3. When the fraction (IV) was treated with TCA, some parts of it was altered into (I) and prolonged treating time completed the alteration as in the case of boiling treatment. The treatment with ethylenediamine tetraacetate did not cause such alteration. In the fractions (II) and (III), the alteration of their chromatographic species into (I) resulted from the same treatment with TCA. The converse alteration, from (I) to the other, has not succeeded as yet.

Digestion of Yeast Cytochrome c by Bacterial Proteinase—Yeast cytochrome c differed from other native proteins such as bacterial α-amylase (*7*, *20*), animal lactic (*8*) and triose phosphate-dehydrogenase (*21*) *etc.* (*22*, *23*), in respect to bacterial proteinase-digestion ; it was rapidly digested by the proteinase without treatment for its denaturation, *i.e.*,

SCHEME 2



heat, acid, or urea treatment, as shown by D in Fig. 4. Treatment with TCA or heat made the cytochrome c more easily digested by the proteinase. This increment of susceptibility to the proteinase-digestion by these treatments was almost proportional to alteration of the chromatographic species from (IV) to (I) by the same treatment; the fraction (IV) suffered alteration into (I) to a large extent by the boiling treatment and by prolonged TCA treatment, and to a small extent by the shortened TCA treatment. On the other hand, cytochrome c was markedly more difficult to be digested in the reduced form than the oxidized form, irrespective of the two kinds of preparation of both forms. Autoxidation and autoreduction were proved not to occur in the reaction course, as shown in Fig. 5.

Effect of TCA and Boiling Treatment on the Enzymic Reduction of Yeast Cytochrome c—Treatment of yeast cytochrome c with TCA or with boiling

Fig. 2. Comparison of chromatographic species of yeast cytochrome c prepared by various procedures.

Figures show the developing patterns of yeast cytochrome c on 2.0×56.0 cm. column of XE-64 resin (100–150 mesh) buffered with $(NH_4)_2HPO_4$-H_3PO_4 buffer and the flow rate was 100 ml. per hour. Columns A, B, C, D, and E correspond to Table II.

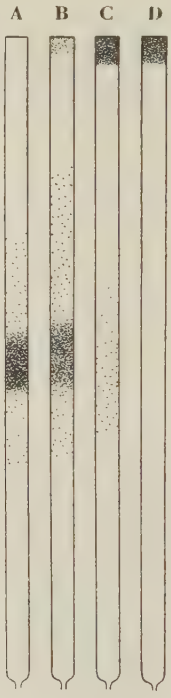


Fig. 3. Effects of various treatments on chromatographic species of yeast cytochrome c.

Column, 1.0×29.0 cm. ; flow rate, 20 ml. per hour ; other conditions were the same as in Fig. 2. For all of these experiments, fraction IV of A in Fig. 2 was used. A, untreated ; B, treated with TCA for 5 minutes at 0.° ; C, treated with TCA overnight at 15° ; D, heated in a boiling water bath for 20 minutes.

changed chromatographic species and digest ability by bacterial pro-

tainase. However, such treatments did not change its reducibility by

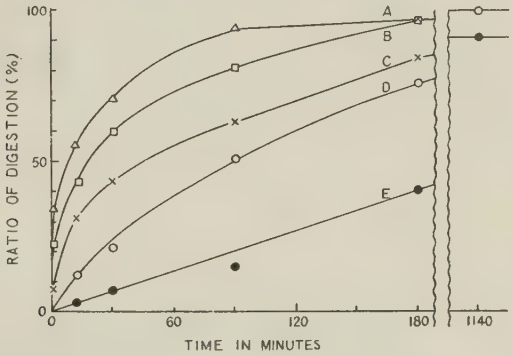


FIG. 4. Digestion course of yeast cytochrome c by bacterial proteinase.

Concentration of cytochrome c in reaction mixture. 0.07 per cent ; concentration of proteinase in reaction mixture, 0.005 per cent ; temperature, 15° ; pH, 7.2 with *M*/20 phosphate buffer. Curves A, B, and C were followed with the samples C, D, and B in Fig. 3, respectively, and D and E were oxidized- and reduced-form of A in Fig. 3, respectively. Difference of extinction at 280 mμ between 0 and 100 per cent in ratio of digestion is approximetely 0.8 in 1.0 cm. of optical length.

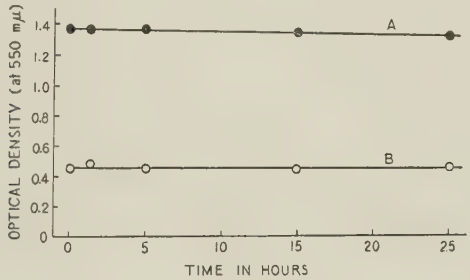


FIG. 5. The courses of autoxidation and autoreduction of yeast cytochrome c.

Experimental conditions were the same as Fig. 4 except that the proteinase was replaced with water. Curves A and B were reduced- and oxidized-form, respectively.

yeast lactic dehydrogenase or by dithionite, as shown in Fig. 6.

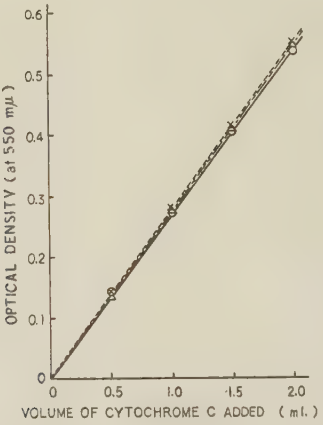


FIG. 6. Effect of TCA and boiling treatment on the enzymic reduction of yeast cytochrome c.

– – × – –, heated in a boiling water bath for 20 minutes; —△—, treated with TCA for 5 minutes at 0° ; —○—, untreated. Concentration of cytochrome c added, 0.07 per cent.

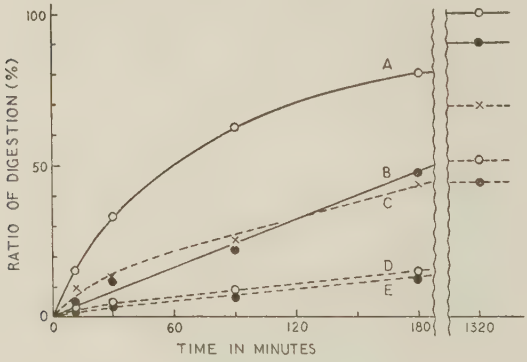


FIG. 7. Comparison of digestion courses between yeast cytochrome c and bovine cytochrome c.

Experimental conditions were the same as Fig. 4, A, oxidized form of yeast cytochrome c ; B, reduced form of yeast cytochrome c ; C, bovine cytochrome c heated in a boiling water bath for 10 minutes ; D, oxidized form of bovine cytochrome c ; E, reduced form of bovine cytochrome c.

Comparison of Digestion by the Bacterial Proteinase between Yeast Cytochrome c and Bovine Heart Cytochrome c—Yeast cytochrome c has been reported (*3*) to have chromatographic species markedly different from the bovine heart cytochrome c, and to have the same specific activity in the " Green brei " system. The bovine and horse heart cytochrome c prepared using TCA were markedly more difficult to be digested by bacterial proteinase even in their oxidized form than yeast cytochrome c in the reduced form, as shown in Fig. 7.

DISCUSSION

Native horse heart cytochrome c shows only one chromatographic species on a cation exchanger (*5*), while baker's yeast cytochrome c shows various kinds of species, each of which is crystallizable in the same crystalline form and has the same biological activity in the test of the succinate-oxygen system, " Green brei ". As the purification of cytochrome c progresses, the use of TCA for the preparation, which seems drastic to the common protein, has been questioned. Boeri and Tosi demonstrated (*24*) that treatment with TCA increases autoxidizability of cytochrome c and they also pointed out (*25*) that it reverses the partially lost biological activity. However, purification of crystalline cytochrome c without the use of such a drastic reagent has not been made as yet. The yeast cytochrome c prepared by the procedure that seems mild to the common protein, not using TCA or ethyl acetate, has a chromatographic species corresponding to the fraction (IV) of Fig. 2 in most parts, while that prepared by the procedure involving TCA treatment has mainly a species corresponding to the fraction (I). It seems likely that the various chromatographic species of yeast cytochrome c is not due to the action of proteolytic enzymes during the purification procedure, since 60 per cent ammonium sulfate extraction does not differ from the 10 per cent ammonium sulfate extraction, though the former extraction should not allow the action of proteolytic enzymes. Indeed, the fraction (IV) changes into the fraction (I) by acid treatment as well as boiling treatment. Artificial, converse change has not succeeded as yet. These facts lead us to the conclusion that the yeast cytochrome c may exist in the living cells in the form of the fraction (IV) but not in that of (I).

The presence of such a variety of chromatographic species in yeast cytochrome c suggests that there are some differences in the constellation (*26*) of protein molecule. In practice, the chromatographic fractions of the yeast cytochrome c are digested by bacterial proteinase in various

rates specific to each of the fractions ; the fraction (I) is markedly more rapidly attacked, as are also the acid- and the boiling-treated ones, than the fraction (IV).

On the other hand, it has been reported (*7*, *8*, *20–23*) that the proteinase can not attack globular proteins such as bacterial α-amylase, alcohol or triose phosphate-dehydrogenase and some other enzymes in their native state but does attack them in their denatured state, because the proteins have special secondary structure in their native state, and their denaturations are caused by the deformation of that structure. However, cytochrome c from various sources, especially from yeast, and some enzymes such as fumarase (*27*) are hydrolyzed by the proteinase even in their native state. Therefore these proteins may exist in a state apt to conjugate with the proteinase, with other substances, or between themselves, like the denatured proteins. The existence of many chromatographic species of yeast cytochrome c different from the mammalian cytochrome c may be explainable from the fact that yeast cytochrome c is more digestable by the proteinase than the mammalian cytochrome c. It seems unlikely that the yeast cytochrome c of essentially different structures is altered to a uniform structure such as the fraction (I), by acid and boiling treatment. However, it may be that there are various modes of existence in the proteins, for example in polymerization, since the yeast cytochrome c is reduced to the same extent by the yeast lactic dehydrogenase and lactate in spite of the differences of chromatographic species.

The reduced form of yeast cytochrome c is less susceptible to the proteinase than the oxidized form. A similar phenomenon has been observed in hemoglobin (*28*) ; several proteins related to it are susceptible to pancreatine in the following order: Globin>methemoglobin> oxyhemoglobin>reduced hemoglobin. It has also been reported (*29*) that reduced hemoglobin differs from oxyhemoglobin in X-ray pattern. From these facts, it is reasonable to conclude that not only heme moiety of cytochrome c but also the secondary structure of protein portion are changed by oxidation and reduction.

SUMMARY

1. Cytochrome c prepared from baker's yeast without using TCA indicates several chromatographic species on cation exchangers.

2. Though yeast cytochrome c is modified by treatments with TCA and heat in respect to chromatographic species and digestability by

bacterial proteinase, its reducibility by yeast lactic dehydrogenase is not changed by these treatments.

3. It was established by the bacterial proteinase method that yeast cytochrome c is more stable to digestion by bacterial proteinase in its reduced form than in its oxidized form.

4. The bovine and horse cytochrome c prepared with the use of TCA are markedly less susceptible to the bacterial proteinase even in their oxidized form than yeast cytochrome c in its reduced form prepared without use of TCA.

Based on these facts, mode of existence of yeast cytochrome c in the living cells was descussed.

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L-GULONIC DEHYDROGENASES IN GUINEA PIG LIVER AND KIDNEY(1)

Sirs:

It has been well established by many investigators that L-xylulose, found in the urine of essential pentosuric humans, is derived from D-glucuronic acid (*2*, *3*). In the case of the guinea pig, L-xylulose appears in urine when glucuronic acid is administered (*2*). However, the details of the enzymatic mechanisms of L-xylulose formation remain obscure. In the present study, guinea pig tissues, which are incapable of synthesising ascorbic acid from glucuronic acid, were employed as the enzyme sources, and it was proved that the liver and kidney contain the DPN- and TPN-specific dehydrogenases of L-gulonic acid. The reaction products were identified as xylulose in the DPN system, and as glucuronic acid in the TPN system, respectively.

Enzyme preparation—Young guinea pigs weighing about 300 g. were sacrificed by decapitation under ether anesthesia. After bleeding, each organ was removed and homogenized with 2.5 volumes of cold isotonic KCl. The homogenate was first centrifuged at 8,500 $\times$ *g*. for 10 minutes, to remove the mitochondria, and the supernatant was centrifuged again at 100,000 $\times$ *g*. for 30 minutes. The protein fraction of the secondary supernatant, which precipitated between 38.5 and 53.5 volume per cent saturation (or 30–50 *w/v* per cent) of ammonium sulfate (pH 7.4), was dissolved in cold glass redistilled water.

Assay and Results—This crude preparation reduced DPN and TPN in the presence of L-gulonate. The same preparation also reduced DPN when D-sorbitol was used as a substrate instead of L-gulonate. But DPN-specific gulonic acid dehydrogenase of liver was remarkably unstable and lost its activity in a few days when stored at $-10°$. This is contrary to D-sorbitol system. For this reason, it is believed that these two reductions are probably due to different enzymes. DPN- and TPN-specific dehydrogenases of L-gulonic acid were active in the preparations of kidney and liver but not in the preparations of cardiac muscle and brain.

In order to accelerate the reaction velocity and to increase the yield of reaction products, DPN reduction by gulonic acid was coupled with

pyruvic acid reduction in the presence of muscle lactic dehydrogenase. Table I shows that the liver enzyme, gulonic acid, DPN, pyruvic acid are indispensable components for gas evolution. In the absence of gulonic acid, insignificant gas evolution took place. Accordingly, it is clear that the gas evolution is not a result of pyruvic acid dismutation. TPN could not replace DPN, and in the TPN system, there was formation of uronic acid, as determined by the color reaction and paper chromatography described below. Similar results were obtained with

TABLE I

Decarboxylation of Gulonate by Liver Enzyme Coupled with Muscle Lactic Dehydrogenase

Each Warburg flask contained 2.0 ml. of liver enzyme, 0.2 ml. of muscle lactic dehydrogenase (*4*), 20 μM Na-gulonate, 20μM Na-pyruvate, 0.5 ml. of 0.1 *M* Tris-buffer (pH 7.4) and 1 mg. of nicotinamide. Side-arm 1 contained pyridinenucleotide (500 μg. of TPN, or 1 mg. of DPN). Side-arm 2 contained 0.3 ml. of 60 per cent perchloric acid. Flasks were shaken at 37°. The gas phase was nitrogen. Pyridine-nucleotides were tipped after temperature equilibration. Gas output was measured for 80 minutes, and then, perchloric acid was tipped. 10 minutes later, again the gas output was measured.

	Gas output in pH 7.4	Total gas output 10 minutes after the tipping of perchloric acid
1. Liver enzyme	31 $\mu l.$	0 $\mu l.$
2. Liver enzyme+TPN	8	0
3. Liver enzyme+DPN	96	205
4. DPN without Na-gulonate	4	0
5. DPN without Na-pyruvate	0	—

the kidney enzyme. Gas evolution observed at pH 7.4 suggests that enzymatic decarboxylation actually occurs, but the possibility of the presence of intermediate such as 3-keto-gulonic acid, proposed by Ashwell (*5*), cannot be excluded at this experimental stage.

Deproteinized reaction mixture of Table I were neutralized with KOH and treated with Norit A at pH 2.0 to remove nucleotides. The supernatant was withdrawn for testing with color reactions. In the case of the supernatant of DPN system (3, Table I), the orcinol reaction spectrum had two absorption peaks at 670 mμ and 540 mμ (Fig. 1),

and the absorption ratio (540 mμ/670 mμ) was 0.45 (*6*). The product

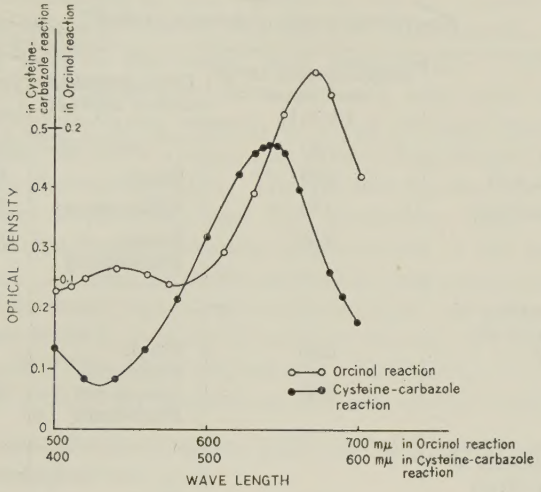

FIG. 1. Adsorption spectrum of orcinol reaction, cysteine carbazole reaction of the reaction product in the DPN system.

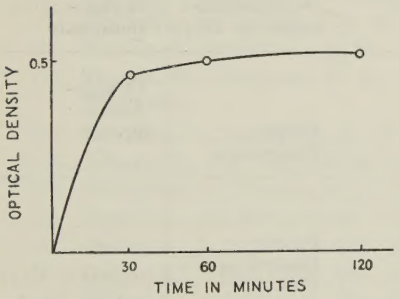

FIG. 2. Time course of color development of cysteine carbazole of the reaction of the reaction product in the DPN system.

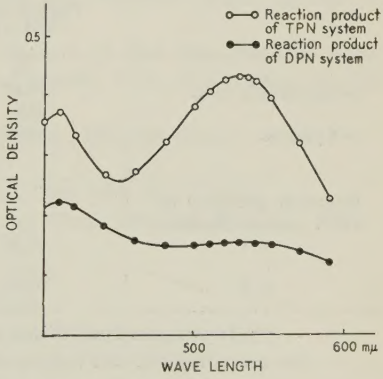

FIG. 3. Carbazole reaction spectrum reaction product.

of the cysteine-carbazole reaction (*7*) showed an absorption maximum at 540 mμ (Fig. 1), characteristic for ketopentoses, and the color density

reached the maximum after 2 hours at 37° (Fig. 2). The carbazole

TABLE II

*Paperchromatography of Reaction Products**

	Benzyl-alcohol acetic acid water (3:3:1)	Orcinol-trichloroacetic acid (*11*)	Aniline-phthalate
	R_f		
D-Glucuronic acid	0.33	Brown	Brown
D-Glucuronolactone	0.59	Yellow-brown	Brown
D-Xylulose†	0.59	Purple Fluorescent§	Brown
Reaction products of DPN system (liver)			
x-1	0.60	Puryle Fluorescent	
x-2	0.50	Gray-green Fluorescent	Brown
Reoction products of TPN system (liver)			
y-1	0.33	Brown	Brown
y-2	0.45	Gray-green Fluorescent	
	Phenol-water (4:1)	Orcinol-trichloroacetic acid	Aniline-phosphate
	R_f		
D-Glucuronic acid	0.32		Orange-Brown
	0.6		
D-Xylulose	0.60	Purple Fluorescent	Brown
Reaction products of DPN system (Kidney)			
x-1	0.59	Purple Fluorescent	
x-2	0.42	Pink	

* Toyo Roshi No. 53 was used as filter paper. Chromatography was ascending one and the front movement was about 300 mm.

† D-Xylulose was synthesized from D-xylose according to Schmidt and Treiber (*12*).

§ White-yellow fluorescence under near ultraviolet light.

reaction (*8*) for uronic acid was negative. On the contrary, in the TPN system, the carbazole reaction for uronic acid was positive (Fig. 3). The

galacturonic acid tests, modifications of the carbazole reaction (*9*) and the cysteine reaction of galacturonic acid (*10*), were both negative.

Paper chromatography was carried out on the lyophilized reaction mixtures. As shown in Table II, the spot x—1 was identified as xylulose, and the spot y—1 as glucuronic acid, respectively. The fluorescent spots other than xylulose and glucuronic acid, seen when orcinol-TCA was used as the developing reagent, have not yet been identified. The reaction product of DPN system was further fractionated by Dowex-1-borate column chromatography. The xylulose fraction was eluted with 0.02 *M* sodium tetraborate, and free L-xylulose ($[\alpha]_D^{22}=+37°$) was obtained from the borate complex after removal of the borate ion as methyl borate. In brief, it can be concluded from manometric data, the response to color reactions and the results of paper- and column- chromatography, that xylulose and uronic acid are the reaction products in the DPN system and TPN system, respectively. Purifications of these enzymes and the study of co-factor resuirements are now under investigation.

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